In vitro modelling of paracrine and endocrine interactions in the human ovary

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Declaration

The studies in this thesis were the unaided work of the author, except where acknowledgement is made by reference. The work described in this thesis has not been previously accepted for, or is currently being submitted for another degree or qualification.

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Abstract

The corpus luteum is one of the most intriguing and understudied endocrine glands in the human body. Understanding the mechanisms behind corpora lutea development, function and regression will enhance many major unresolved issues in human reproduction, with important consequences on infertility, miscarriage and contraception. Formed at the end-stage of folliculogenesis, the corpus luteum has the important role of being the interface between pregnancy and menstruation. This transition involves the terminally differentiated granulosa- and theca-lutein cells along with endothelial, fibroblast and immune cells to form the most steroidogenically active tissue in the body. In a non-conception cycle the corpus luteum will experience high cell turnover, intense remodelling and structural and functional demise within a 14-day period, succinctly followed by the initiation of a new cycle. Alternatively, during maternal recognition of pregnancy, the corpus luteum will continue to secrete vast quantities of steroid hormones under the trophic stimulation of conceptus derived-human chorionic gonadotrophin (hCG), subsequently maintaining structural and functional integrity. Therefore the luteal lifespan is regulated by massive tissue and vascular remodelling that is induced and regulated by gonadotrophins (luteinising hormone (LH) and hCG) which elicit disparate changes in the number and function of cells that do not express hCG/LH receptors. For example, luteolysis in women is associated with an up-regulation of the expression and activity of matrix metalloproteinase-2 (MMP-2) which is inhibited by hCG during maternal recognition of pregnancy. As the primary source of MMP-2 is fibroblasts that do not express LH/hCG receptors, the scope of this thesis was to investigate the regulation of MMP-2 by dissecting out potential paracrine signalling molecules.

Women with regular cycles having hysterectomy for non-malignant conditions and women undergoing oocyte retrieval for assisted conception were used in the current study. Novel primary cultures and co-cultures of luteinised granulosa cells and fibroblast-like cells allowed the mechanistic *in vivo* interactions of the corpus luteum to be mimicked with an *in vitro* system. Herein, activin A is identified as a regulated molecule that may promote tissue remodelling during luteolysis. Activin A is secreted by luteal steroidogenic cells and at physiological concentrations it up regulates MMP-2 activity and expression in luteal fibroblast-like cells. HCG can inhibit activin A action through several mechanisms including up-regulating activin inhibitors, inhibin A and follistatin.

If activin A is a paracrine molecule with potential roles in luteolysis, cortisol is a paracrine molecule with possible roles in luteal rescue. The corpus luteum expresses glucocorticoid receptors and the cortisol metabolising enzymes 11β hydroxysteroid dehydrogenase type 1 (11βHSD1) and type 2 (11βHSD2). Both *in vitro* and *in vivo,* hCG promotes 11βHSD1 expression and facilitates the local generation of cortisol. Cortisol at physiological concentrations inhibits local MMP-2 expression to inhibit the tissue remodelling associated with luteolysis. During luteolysis activin and hCG have opposing effects and the same is true during luteinisation. HCG promotes a luteinised granulosa cell phenotype, whilst activin A promotes a more follicular phenotype by up-regulating granulosa cells markers such as follicle-stimulating hormone (FSH) receptor, 11βHSD2 and blocking hCG up-regulation of steroidogenic acute regulatory protein (StAR), 3β hydroxysteroid dehydrogenase (3βHSD) and 11βHSD1. Activin A appears to be an anti-luteal molecule in nature. Activin A is not the only activin produced by the corpus luteum, activin B is also a luteal cell product and its secretion is regulated in a similar manner to activin A with similar endocrine effects. As activin has a fundamental role at beginning and end of the luteal phase, it has the potential to be a major regulatory molecule in women. Indeed it can regulate its own activity in the absence of hCG by self promoting its own expression and down regulating its own inhibitors.

Collectively these results suggest that activin is an excellent anti-luteal molecule whose paracrine actions are to remove or potentially inhibit luteal tissue formation, and moreover to facilitate human luteolysis. However, the biological actions of activin A are inhibited during maternal recognition of pregnancy by the endocrine actions of conceptus-derived hCG that has marked and disparate changes on surrounding cell types that do not expression the LH/hCG receptor. Consequently, luteolysis is prevented, such that luteal fibroblast-like MMP-2 is inhibited as hCG-derived cortisol supports promotes the maternal recognition of pregnancy.

Presentations and Publications relating to this thesis

Chapter 3: *In vitro* evidence suggests that activin A may promote tissue remodelling associated with human luteolysis

Paper

Myers, M., Gay, E., McNeilly, A.S., Fraser, H.M., and Duncan, W.C. (2007) *In vitro* evidence suggests that activin A may promote tissue remodeling associated with human luteolysis. *Endocrinology* **148**, 3730-9

Abstracts

Duncan, W.C., Gay, E., Fraser, H.M. and **Myers, M.** (*SSR July 2006*- Invited Oral Presentation)
Epithelial and stromal interactions in the regulation of luteal tissue remodeling

Myers, M., Gay, E. and Duncan, W.C. (*SRF July 2006* – Oral Presentation)
Does hCG inhibit luteal MMP-2 by increasing follistatin?

Myers, M., Gay, E. and Duncan, W.C. (*Munro Kerr Feb 2006*- Oral Presentation)
Evidence that Activin A is a paracrine regulator of tissue remodelling in the human corpus luteum

Myers, M., Gay, E. and Duncan, W.C. (*British Fertility April 2005*- Oral Presentation)
ActivinA as a paracrine regulator of human luteal MMP-2 expression

Chapter 4: Role of glucocorticoid metabolism during maternal recognition in women

Paper

Myers, M., Lamont, M.C., van den Driesche, S., Mary, N., Thong, K.J., Hillier, S.G. and Duncan, W.C. (2007). Role of luteal glucocorticoid metabolism during maternal recognition of pregnancy in women. *Endocrinology* **148**, 5769-79

Abstracts

Myers, M., Lamont, M.C., Mary, N. and Duncan, W.C. (*British Fertility April 2007*- Oral Presentation)
Glucocorticoid metabolism is hormonally regulated in human luteal cells

Myers, M., Lamont, M.C., Thong, K.J., Fraser, H.M. and Duncan, W.C. (*SSR July 2006*- Poster Presentation)
Is cortisol a paracrine regulator of the human corpus luteum?

Chapter 5: Activin A reduces the luteinisation of human luteinised granulosa cells and has opposing effects to hCG *in vitro*

Paper

Myers, M., van den Driesche, S., McNeilly, A.S. and Duncan, W.C. (2007). Activin A reduces luteinisation of human luteinised granulosa cells and has opposing effects to human chorionic gonadotrophin (hCG) *in vitro*. *Human Reproduction (Submitted)*

Abstract

Myers, M., McNeilly, A.S. and Duncan, W.C. (*SSR July 2007*- Oral Presentation) Activin A reduces luteinization of human granulosa cells

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Abbreviation	Definition
11βHSD1	11β hydroxysteroid dehydrogenase
17βHSD	17β hydroxysteroid dehydrogenase
3βHSD	3β hydroxysteroid dehydrogenase
αSMA	alpha smooth muscle actin
ACTH	adrenocorticotrophic hormone
ActRII	activin receptor type II (A)
ActRIIB	activin receptor type IIB
ADAMTS1	a disintegrin and metalloproteinase with thrombospondin motifs
ALK	activin like-kinase
AMP	adenosine-monophosphate
ANOVA	analysis of variance
ATP	adenosine-triphosphate
βgly	beta glycan
BMP	bone morphogenic protein
BSA	bovine serum albumin
cAMP	cyclic AMP
CBP	cortisol binding protein
cDNA	complimentry DNA
COC	cumulus oocyte complex
COREC	common reproductive ethics committee
Co-Smad	common Smad
CTGF	connective tissue growth factor
Cx37	connexin 37
Cx43	connexin 43
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylendiaminetetraacetic acid
EFREC	Edinburgh Fertility Reproductive Endocrine Clinic
ELISA	enzyme-linked immunosorbent assays
ER	oestrogen receptor
FBS	fetal bovine serum
FS	follistatin
FSH	follicle-stimulating hormone
FSHR	follicle-stimulating hormone receptor
G6PDH	glucose-6-phosphate dehydrogenase
GDF9	growth and differentiation factor 9
GPCR	G protein coupled receptor
GR	glucocorticoid receptor
hCG	human chorionic gonadotrophin
HDL	high density lipoprotein
HPG	hypothalamic pituitary gonadal axis
HRP	horseradish peroxidase
HSP	heat shock protein
IgG	immunoglobulin G
I-Smad	inhibitory Smad
ITS	Insulin, transferrin, selenious acid

IVF	*in vitro* fertilisation
LDL	low density lipoprotein
LGC	luteinised granulosa cell
LH	luteinising hormone
LHR	luteinising hormone receptor
MCP-1	macrophage chemotractant protein-1
MMP	matrix metalloproteinase
MMP-1	matrix metalloproteinase-1
MMP-2	matrix metalloproteinase-2
MMP-9	matrix metalloproteinase-9
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MT1-MMP	membrane type- matrix metalloproteinase
NADPH	nicotinamide-adenine dinucleotide phosphate
NAD+	nicotinamide-adenine dinucleotide
NDS	normal donkey serum
NGS	normal goat serum
NRS	normal rabbit serum
$P450_{arom}$	cytochrome P450 aromatase
$P450_{scc}$	cytochrome P450 side chain cleavage
PAP	peroxidase anti-peroxidase
PBS	phosphase buffered saline
PCR	polymerase chain reaction
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PRL	prolactin
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RNAi	ribonucleic acid interference
R-Smad	receptor mediated Smad
RT	reverse transcription
SARA	Smad anchor for receptor activation
SCP	sterol carrier protein
SDS	sodium dodecyl sulfate
Smad	*sma* gene and drosophila mothers against decapentaplegic
StAR	steroidogenic acute regulatory protein
TAE	tris-acetae-EDTA
TBS	tris buffered saline
TGFβ	transforming growth family beta
TIMP	tissue inhibitor of matrix metalloproteinase
TLC	theca lutein cell
Tm	annealling temperature
TNFα	tissue necrosis factor alpha
TSA	tyramide signal application
TSH	thyroid stimulating hormone
VEGF	vascular endothelial growth factor
ZP	zona pellucida

1 Literature review

The ovary is one of the most dynamic organs in the human body, experiencing extensive tissue remodelling each month in a cyclic manner. Within the ovary is the most steroidogenic gland in the body, the corpus luteum. Formed at the end-stage of folliculogenesis from the dominant follicle, the corpus luteum is of fundamental importance in reproductive biology. It has the principal role of preparing the uterine environment for implantation of a conceptus by secreting vast quantities of steroid hormones. In the absence of conception, the functional lifespan of the corpus luteum is approximately 14 days, after which it will undergo the process of luteolysis. This process involves demise of the gland whereby there is a loss of structural (tissue) and functional (hormone synthesis) integrity that consequently leads to endometrial shedding. During a conception cycle however, the structural and functional integrity of the corpus luteum is maintained in the presence of an implanting conceptus which secretes logarithmically increasing amounts of hCG. Consequently, luteolysis is inhibited and the corpus luteum will maintain the role of principle steroid production until the luteo-placental shift.

Understanding the molecular mechanisms behind processes such as luteolysis and luteal rescue during maternal recognition of pregnancy provides valuable insights toward reproductive promotion and prevention. This thesis aims to dissect out possible molecular pathways in corpus luteum physiology in order to further understand the changes in multiple cells types, angiogenesis, proliferation and cell death that is both unique yet predictable. Therefore, as well as increasing knowledge about the corpus luteum itself, these studies can facilitate the understanding of basic mechanisms involved in normal and dysregulated fundamental tissue processes. Examples of important physiological and pathological processes include integral remodelling events such as wound repair and tumourigenesis.

Formation of the corpus luteum can only occur after successful folliculogenesis. Therefore the review of the current literature will begin with follicle formation involving the regulation of gonadotrophins, follicle selection and feedback, consequently leading to the oestrogenic dominant follicle that undergoes the process of ovulation to form the focus of the current thesis, the corpus luteum.

1.1 The ovary

The ovaries are paired intra-abdominal organs that have the principal roles of producing (i) the female gamete, the ovum and (ii) and sex steroid hormones, notably oestrogen and progesterone. Each month, the ovary undergoes changes that are tightly orchestrated by growth factors, gonadotrophins and sex steroids in a cyclic manner. The ovarian cycle represents the growth and regression of follicles from a primordial status to post-ovulatory corpora lutea (Figure 1.1), with ovulation occurring approximately every 28 days in woman. Although the ovulatory process generally occurs once a month, the process of folliculogenesis (the activation and development of a primordial follicle through to ovulation) is reported to take approximately four or more months in women (Gougeon, 1986).

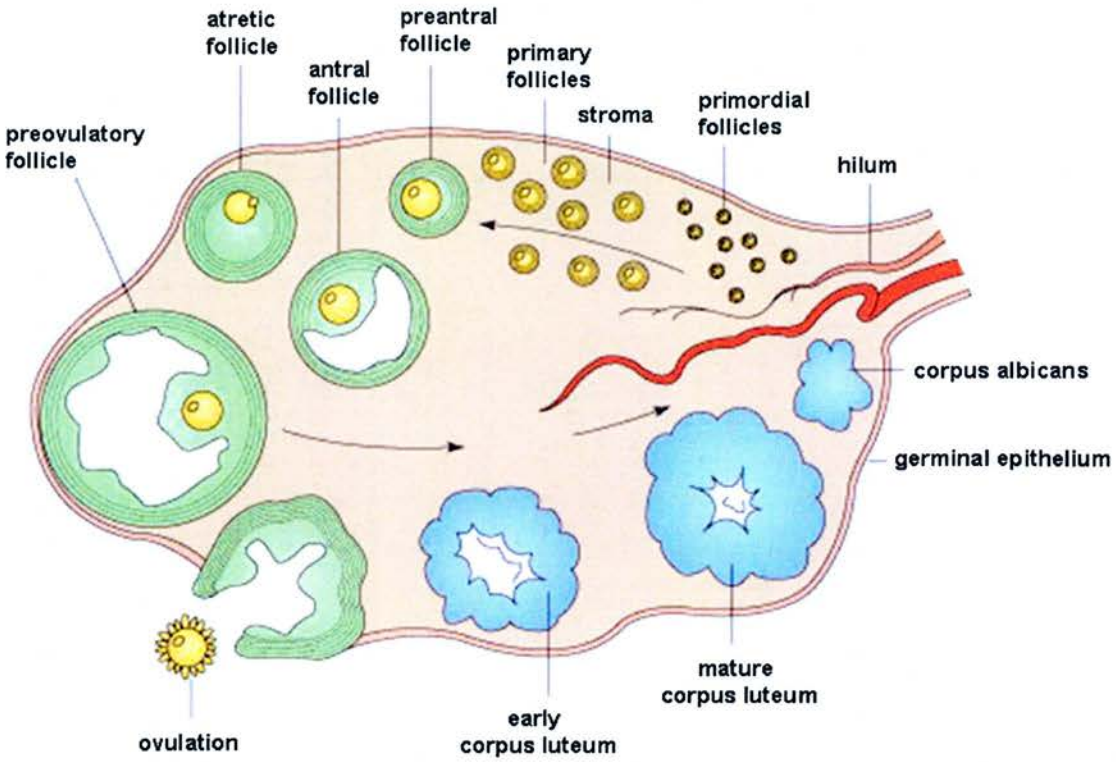


Figure 1.1 Schematic diagram of the ovary demonstrating the various follicle and cell types. Folliculogenesis begins with the most primitive follicle, the primordial which undergoes various stages of development under the influence of growth factors, gonadotrophins and steroid hormones to mature into a pre-ovulatory follicle that is capable of ovulation. The resulting follicular cells undergo luteinisation to form the steroidogenically active corpus luteum that has the integral role of maintaining early pregnancy.

(Porth, 2004)

1.1.1 Germ cells

Unlike the male whereby new germ cells are produced throughout life, a female is born with (human, mouse) or acquires shortly thereafter (rat, pig) a finite number of germ cells. Early studies suggest that the peak number of germ cells is observed at the time of mitotic to meiotic transition, which in the human is around 20 weeks of gestation with the number of primary oocytes reaching a maximum of 6-7 million (Baker, 1971) (Figure 1.2). By birth however, this number has markedly declined to 1-2 million and by the time of puberty Gougeon estimated it to have further fallen to a mere 400,000 (Gougeon, 1986). Of the original 6-7 million germ cells that were originally produced in the pair of fetal ovaries, only about 400 will reach ovulatory status, therefore in women more than 99% of ovarian follicles will undergo atresia in postnatal life (Baker, 1971; Hsueh *et al.*, 1994).

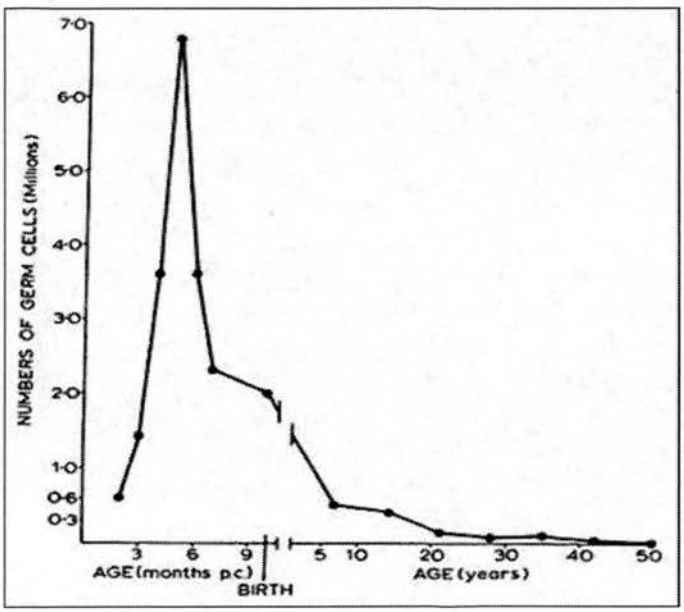


Figure 1.2 In the human all primordial follicles are formed in the fetus between 6 and 9 months gestation. There is a marked loss of oocytes during this period and consequently the number of primordial follicles decreases due to apoptosis and follicle recruitment. Follicle loss continues throughout reproductive life until very few, if any, are present after menopause.

(Baker, 1971)

It is worth noting that during the past three years considerable amount of controversy has arisen regarding the dogma on a 'finite' germ cell pool in women. Studies thus far have concentrated on mouse models beginning with the initial study from Johnson *et al.,* whereby it was reported that the existence of germline stem cells and therefore follicular renewal may exist in the postnatal mouse ovary (Johnson *et al.*, 2004). As expected, this contrast in doctrine raised significant controversy and was subsequently followed by letters of criticism (Telfer *et al.*, 2005) and follow up studies (Eggan *et al.*, 2006; Kerr *et al.*, 2006) including one from the initial laboratory (Johnson *et al.*, 2005). Consequently, no further conclusions regarding this matter have been published of late and, until more work is focussed upon this issue, the 'finite germ cell population' theory in a female gonad remains.

1.1.2 The ovarian cycle

The ovarian cycle may be split up into three components, the pre-ovulatory follicular development, ovulation and the functional life span of the corpus luteum (Hillier, 1994b). Each of these functional stages are characterised and directed by defined key proteins in which transgenic knockout mouse models have proved to be of great importance (Matzuk and Lamb, 2002). The next few sections will review folliculogenesis (gonadotrophin-independent and gonadotrophin-dependent) and ovulation before discussing the functional lifespan of the corpus luteum.

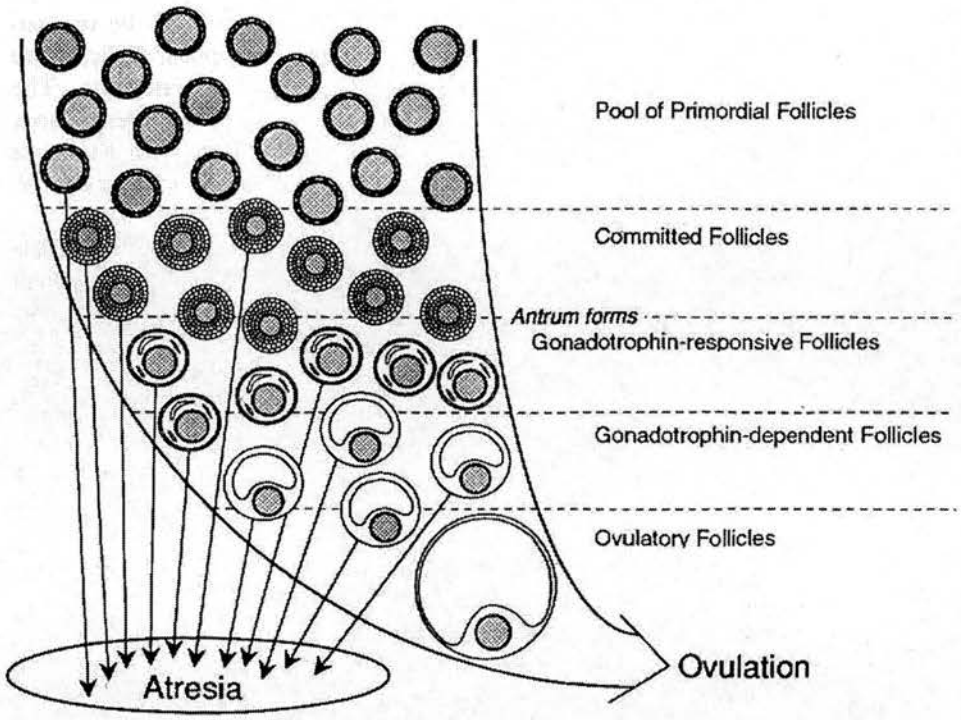


Figure 1.3 A functional model of follicular growth and atresia that describes the gonadotrophin-independent and gonadotrophin-dependent events. The quiescent primordial follicle pool experiences little atresia and activated follicles move into the growth phase to become committed follicles. Once the follicle forms an antral cavity it becomes gonadotrophin-responsive and will eventually become gonadotrophin-dependent. High rates of atresia are evident at this stage and in the absence of gonadotrophic support follicle development will cease. In the presence of high FSH concentrations the dominant follicle will express LH receptors and in response to a LH stimulatory surge this follicle will undergo ovulation.

(Scaramuzzi *et al.*, 1993)

1.1.2.1 Folliculogenesis

The process of folliculogenesis involves the co-ordinated growth and development of primordial follicles through to a dominant follicle capable of ovulation. This can be further classed into either gonadotrophin-independent or gonadotrophin-dependent events (Figure 1.3). Early follicle development involves the activation of primordial follicles into the growth phases. Anatomically, primordial follicles consist of an oocyte partially or fully surrounded by squamous pre-granulosa cells (Figure 1.4). Once in the growing pool, the transition of a primordial into a primary follicle is associated with moderate oocyte growth and morphological transformation of previously flattened squamous granulosa cells into cuboidal cells (Hirshfield, 1991). Although the primordial follicles are formed during fetal life, they may remain dormant within the ovaries for as long as 40 years (Zeleznik and Pohl, 2006). Transcription factors such NOBOX (Rajkovic *et al.*,

2004), Foxl2 (Schmidt *et al.*, 2003)and Foxo3a (Castrillon *et al.*, 2003) have been shown to be critical for early follicle formation in transgenic mice and mutations in Foxl2 and NOBOX have been linked to premature ovarian failure in women (Gersak *et al.*, 2004; Pangas and Rajkovic, 2006). Consequently, the aforementioned mouse gene knockout studies, coupled with many others (Matzuk and Lamb, 2002; Rajkovic and Matzuk, 2002) have provided new insights into factors that may be involved in recruitment of follicles into the growing pool.

The transition of the primordial follicle to a primary follicle marks its entry into the growth phase. Morphologically this transition is characterised by a transformation of squamous pregranulosa cells into a more cuboidal phenotype and this is accompanied by the formation of a zona pellucida (ZP) matrix. The ZP is a non-cellular membrane composed of three glycoproteins (ZP1, ZP2 and ZP3) (Harris *et al.*, 1994) that forms between the primary oocyte and granulosa cells. This matrix provides an effective means for selective transfer of nutrients to and from the oocyte (Centola, 1999) and a selective barrier to polyspermy at fertilisation after ovulation.

More recently, many investigators have taken to studying oocyte-specific proteins which have been found to be imperative for successful folliculogenesis. An example of an oocyte-specific gene which is essential for follicular development is growth differentiation factor 9 (GDF9). This factor is expressed in oocytes beginning approximately at birth and continuing through the late antral stage in adults (Dube *et al.*, 1998; Elvin *et al.*, 1999b; Rajkovic *et al.*, 2004). One of the critical roles of GDF9 appears to be post-primary follicle development. Homozygous mice for the GDF9 deletion arrest at the primary follicle stage with a single layer of granulosa cells, do not develop a thecal layer, and have defects in oocyte meiotic competence and growth (Dong *et al.*, 1996). Furthermore sheep homozygous for GDF9 mutations are sterile due to premature ovarian failure (McNatty *et al.*, 2006) suggesting that this gene is critical in both mono- and polyovular species.

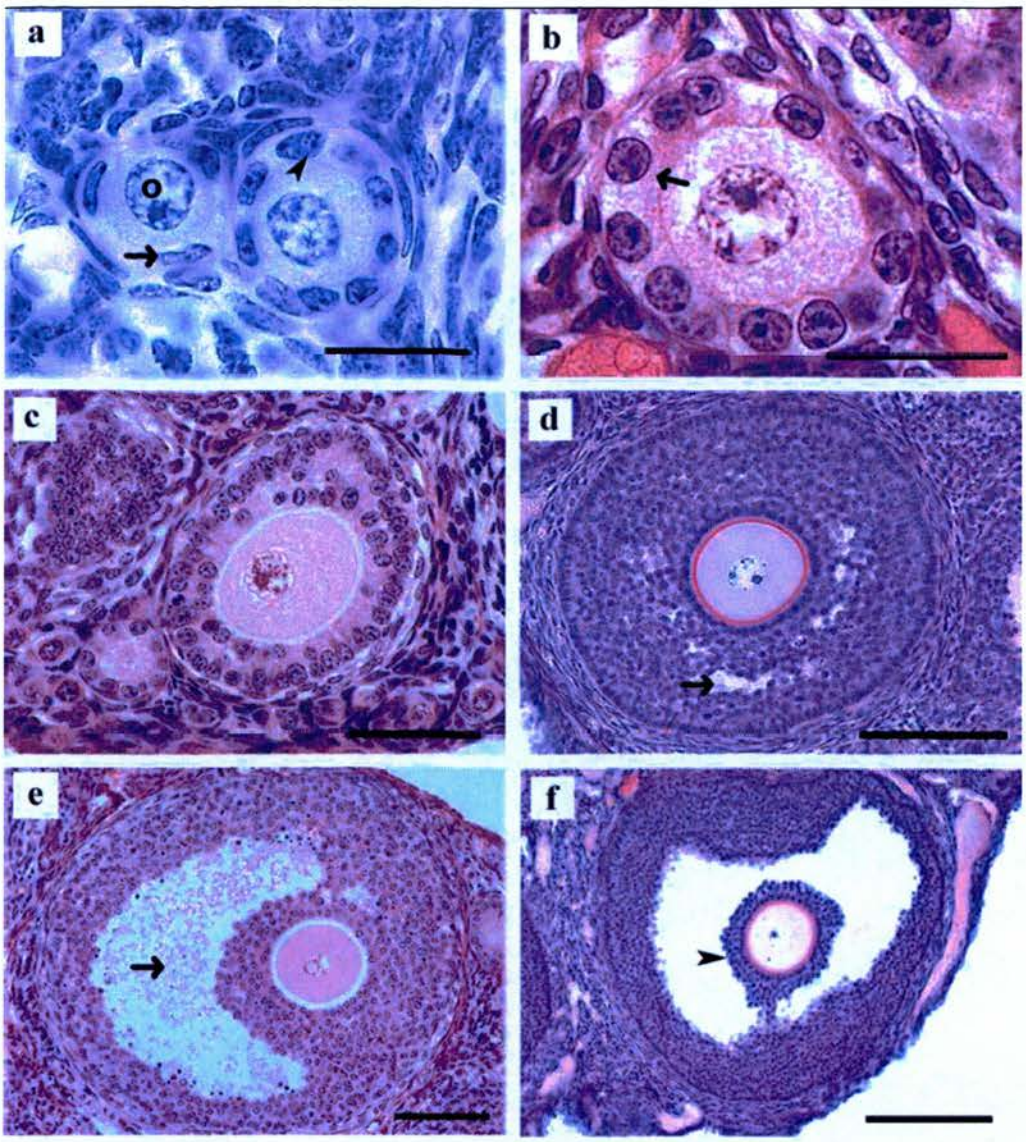


Figure 1.4 Morphological classification of folliculogenesis. These images are taken from the mouse ovary, however the general morphology is the same as the human with the exception of the size of the follicles; **a,** Primordial follicles consists of an oocyte (o) either partially or fully surrounded by a layer of squamous pregranulosa cells (arrow). As these follicles enter the growth phase the transformation of squamous granulosa cells into cuboidal granulosa cell (arrowhead) is morphologically evident. **b,** The primary follicle is composed of a single layer of cuboidal granulosa cells (arrow) whilst **c,** a secondary follicle is continuing to acquire more layers of granulosa cells. **d,** As the follicle acquires many layers of granulosa cells the emergence of an antral cavity (arrow) is evident. **e,** A fully formed antral follicle possesses a fully formed fluid filled cavity (arrow). At this stage the antral follicle is gonadotrophin-dependent and will experience atresia if 'threshold' FSH levels are not sufficient. **f,** The pre-ovulatory follicle is the largest type and a clear distinction can be made between the cumulus granulosa cells (arrowhead) and the mural granulosa cells on the follicle wall. Scale bars a-b=20μm, c=50μm, d-e=100μm, f=200μm

(Myers *et al.*, 2004)

As the granulosa cells begin to proliferate, form multiple layers and the oocyte enlarges, the primary follicle undergoes the progression to a secondary/pre-antral follicle. Development of a secondary follicle is also associated with the recruitment of an additional outermost cellular layer that surrounds the highly dividing granulosa cell layers. These cells are termed theca cells and are derived from the inter-follicular stromal tissue. The theca cell layer comprises the two outermost layers of the secondary follicle. The theca interna layer is comprised of elongated endocrine cells and is approximately 3-5 cells thick. This layer is highly vascularised and lies immediately adjacent to the basal lamina that separates the theca and granulosa cells. Adjacent to the theca interna layer is an outermost layer of non-steroidogenic theca cells (theca externa) that are loosely organised between the theca interna and inter-follicular stroma (Magoffin, 2005).

Unlike granulosa cells, not a lot is known about theca cells, however they have been reported to have differentiated from the unspecialised mesenchymal ovarian stroma (Magoffin, 2005). Interestingly, the granulosa cells are believed to play a key role in the differentiation of theca cells. Unidentified small molecular weight proteins secreted from granulosa have been confirmed to stimulate theca cell differentiation, under the control of FSH (Magoffin and Magarelli, 1995). It is generally assumed that such precursor cells are capable of proliferation and differentiation and consequently have properties common to that of stem cells (Magoffin, 2005), however more research in this area is warranted.

Absolutely imperative for the survival of a multilayered follicle is an extensive network of communication portholes known as gap junctions (Hirshfield, 1991). Formed from intercellular membrane channels, gap junctions serve the importance of allowing nutrients, inorganic ions, second messengers and small metabolites transfer from cell to cell. This serves an important role, particularly as the multilayered follicle contains an oocyte surrounded by many layers of avascular granulosa cells. Much ovarian research has focussed upon the importance of gap junctions with a family of proteins called connexins receiving a lot of attention. Connexins are intercellular channel-forming membrane proteins that elicit cell-to-cell communication. They have been shown to be absolutely imperative for follicular development to the pre-antral stage. Of particular relevance for ovarian cellular communication has been the discovery of connexins 37 (Cx 37) and 43 (Cx 43) (Albertini *et al.*, 2001). Studies using mice null for either Cx 37

(oocyte-granulosa cells junctions) or Cx 43 (granulosa-granulosa cell junctions) have provided clear evidence that these types of gap junctions are indeed essential for follicular development beyond the pre-antral stage (Ackert *et al.*, 2001; Simon *et al.*, 1997). As the pre-antral follicle acquires six to seven layers of granulosa cells, a fluid filled antral cavity (filled with nutrients, electrolyte, water, growth factors and steroid hormones) is generated within the granulosa cell layers and marks the formation of an antral follicle. Based on measurements of mitotic indices and granulosa cell doubling times, the growth of a follicle to the antral stage is estimated to take at least 85 days in women (Gougeon, 1986).

As the antral cavity begins to develop the follicle acquires gonadotrophin responsiveness and it is at this stage of development that most follicles will undergo the process of atresia, with the remaining only surviving with sufficient gonadotrophic support. For this reason, any early antral follicles before the onset of puberty will subsequently undergo atresia as blood concentrations of follicle-stimulating hormone (FSH) do not rise sufficiently to sustain their development (Hillier, 1994a). However after puberty as each menstrual cycle begins, FSH concentrations rise beyond the critical threshold and consequently follicles are recruited for pre-ovulatory development. Key studies utilising hypophysectomised and pre-pubertal monkey ovaries have elegantly shown that gonadotrophic stimulation is essential for follicular development past the early antral stage (Knobil *et al.*, 1959; Peters, 1992). These early studies coupled with the many more modern transgenic mouse models of gonadotrophin deficiency clearly demonstrate that in the absence of either FSH/luteinizing hormone (LH) ligand or receptor, folliculogenesis is compromised and infertility results (Abel *et al.*, 2000; Danilovich *et al.*, 2000; Dierich *et al.*, 1998; Kumar *et al.*, 1997; Lei *et al.*, 2001; Zhang *et al.*, 2001). Therefore developmental competence of ovarian follicles after antral cavity formation is known to be a gonadotrophin-dependent event.

The stimulation of granulosa cells by FSH results in important co-ordinated changes in the activation of numerous genes whose products are responsible for the acquisition of steroidogenic capacity and their ability to respond to LH (Hillier, 1991a). One of the hallmarks of ovarian follicle development is the acquisition of aromatase cytochrome P-450 ($P450_{arom}$) and its encoded protein which results in the ability of the follicle to produce oestrogen as described in section 1.1.3. During each cycle one follicle assumes

dominance, secretes oestrogen and ovulates, whilst the others become atretic (Gougeon, 1986; Hillier, 1994a; Hillier *et al.*, 1994) (Figure 1.3). This selection of the dominant follicle is a result of the developmental changes in FSH and LH responsiveness which leads to gonadotrophic control of ovarian steroid secretion.

1.1.3 Gonadotrophic control of ovarian steroid secretion

Described by Eli Adashi as the 'master gland' of steroid production in reproductive biology, the ovary is indeed an extremely steroidogenically active endocrine gland. Mature granulosa cells are the cellular source of the two most important ovarian steroids, oestradiol and progesterone. Whilst the production of progesterone is possible by single cell components alone (ie. theca cells and granulosa-lutein cells), the biosynthesis of oestrogen requires the co-operation between granulosa cells and the adjacent thecal cells (Adashi, 1994). Proposed by Falck in 1959 and later confirmed by Ryan and Petro the two-cell, two-gonadotrophin theory provides evidence that the thecal interstitial cells are the producers of C_{19} androgens (Falck, 1959; Ryan and Petro, 1966). The androgens can then cross the basal lamina to diffuse into the granulosa cells where they aromatised into C_{18} oestrogens, which are essential for complete follicular growth and development (Figure 1.5). Both the theca and granulosa cells are responsive to the gonadotrophins, LH and FSH respectively. The delivery of LH to the theca cells leads to the synthesis and secretion of androstenedione whilst in response to FSH, the $P450_{arom}$ enzyme in the granulosa cells will aromatase androstenedione to oestrone, which then is converted to oestradiol by 17β hydroxysteroid dehydrogenase (17βHSD).

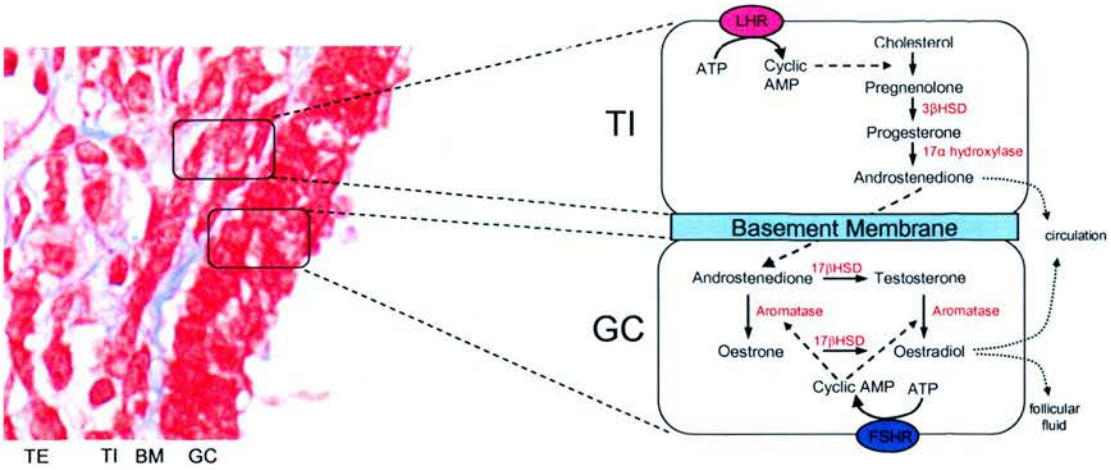


Figure 1.5 The two-cell, two-gonadotrophin concept of ovarian steroid secretion demonstrates that follicular oestradiol synthesis is primarily under the influence of the gonadotrophins. The dominant follicle is consists of an outermost theca externa (TE) layer, an inner theca interna (TI) cell layer that produces androgens that cross the basement membrane (BM) to enter the granulosa cell (GC) layer whereby they are aromatised to oestrogens which are secreted into either the circulation or follicular fluid. LHR- LH receptor, ATP- adenosine-triphosphate, AMP-adenosine-monophosphate, FSHR- FSH receptor.

Oestrogen secreted by the pre-ovulatory follicle is vital to female reproductive physiology, serving to prepare the reproductive tract for conception, suppressing FSH pituitary release to minimise the likelihood of multiple dominant follicles, and to cause the release of the mid-cycle LH surge from the pituitary gland to trigger ovulation (Hillier, 1990; Hillier *et al.*, 1994).

1.1.4 Follicle selection

Before successful ovulation can proceed, selection of the dominant follicle must occur. In most species, including humans the development of large ovulatory sized follicles is not a random process (Fortune, 1994). These follicles do not develop during the luteal phases (as discussed in section 1.2), however a cohort of smaller follicles emerge during the early follicular phase. In mono-ovulatory species such as women, only one of these follicles will continue to grow during the late-follicular phase whilst the rest of the cohort will regress (Fortune, 1994). This follicle is termed the 'dominant follicle' and grows faster than the rest of the cohort by producing higher concentrations of oestrogens and inhibin (Zeleznik and Pohl, 2006). The exact reasons why one follicle can emerge as

dominant are not fully understood, however it strongly believed that one follicle becomes more sensitive to FSH than the other (Fauser and Van Heusden, 1997). The dominant follicle will then proceed to produce ostrogens and inhibins in order to suppress FSH released during the mid-follicular phase. Consequently, the other antral follicles become deprived of FSH stimulation that is essential for survival and undergo apoptosis (Hillier, 1994a; Zeleznik and Pohl, 2006), leaving only the dominant follicle to experience ovulation. Elegant monkey studies have shown that neutralisation of circulating oestrogens in the follicular phase leads to maintained FSH levels in the circulation, thus leading to the development of multiple pre-ovulatory follicles (Zeleznik and Pohl, 2006).

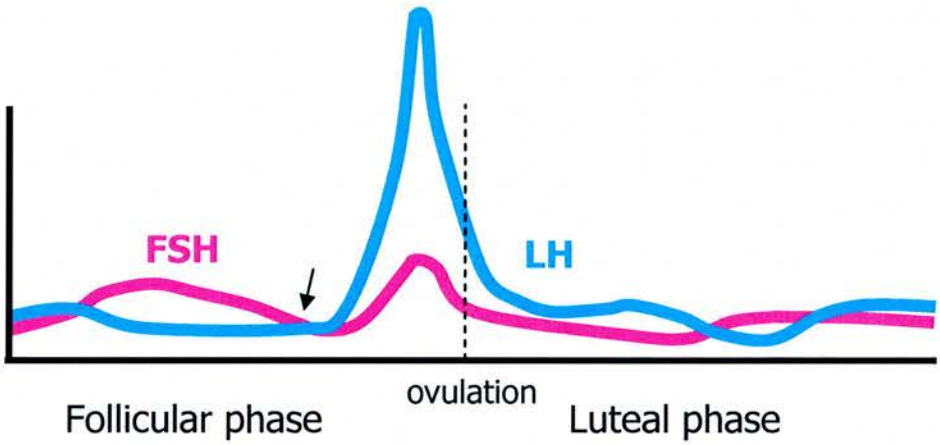


Figure 1.6 Pattern of gonadotrophic secretion across the ovarian cycle. Note the fall in FSH during follicular selection (arrow)

1.1.5 Ovulation

Ovulation is a complex, inflammation-like, and LH-induced process that permits that release of a fertilisable oocyte (Espey, 1980; Richards, 2005). The only follicles capable of releasing an oocyte for fertilisation are those of a pre-ovulatory status. As described above, both LH and FSH are required for sex steroid synthesis, however once the follicle has reached a pre-ovulatory status FSH is thought to induce cell surface receptors for LH on granulosa cells. Consequently in pre-ovulatory follicles LH can regulate both thecal cell androgen production and granulosa cell androgen aromatisation. It has been proposed that the acquisition of LH receptors may serve to protect the follicle from the falling plasma FSH concentrations by providing the follicles with the ability to respond to LH (Zeleznik and Pohl, 2006). Ovulation of the oocyte-cumulus complex is induced by LH from the pituitary gland in response to increasing ovarian steroid synthesis from the

pre-ovulatory follicle. This next stage of the ovarian cycle, ovulation can be split up into three main components; oocyte reactivation, follicular rupture and luteinisation.

1.1.5.1 Oocyte reactivation

Oocytes undergo spontaneous maturation in response to the LH surge, which consequently results in prophase-arrested oocytes making the transition from G2 phase of the cell cycle to M phase. In addition removal of the oocyte from somatic cell contacts *in vitro* results in spontaneous maturation (Dekel and Beers, 1978; Dekel and Beers, 1980). This is thought to be a result of a decline in intracellular cyclic adenosine monophosphate (cAMP) which the oocyte itself naturally contains very little. Maturation can therefore be prevented by the addition of cAMP which is most likely derived from the granulosa cells. Oocytes do not contain gonadotrophin receptors and therefore reactivation of the oocyte in response to LH must depend upon signals from cells that do express the LH receptor. Such LH responsive cells (ie granulosa cells) must signal to the oocyte in order to maintain it in an arrested state. However in event of the LH surge, LH acts via the LH/ hCG receptor to increase intracellular cAMP concentration (which at such high concentrations) target the granulosa cells and break down intercellular communications, subsequently uncoupling the oocyte and resulting in a reduction in oocyte cAMP (Albertini and Anderson, 1974). Once the oocyte re-enters the cell cycle it is no longer arrested in diplotene stage of meiosis I. However, this does not last long and soon after it will rearrest again at the metaphase II stage until fertilisation occurs.

1.1.5.2 Follicular rupture

Follicular rupture involves the extrusion of the cumulus-oocyte complex (COC). Like resumption of meiotic competence, this stage is dependent upon the LH surge, however it may well begin 5-6 days prior (Adashi, 1994). Rupture of the follicular wall and extrusion of the COC is accompanied by a gentle, rather than an explosive expulsion of the oocyte and antral fluid like initially thought (Adashi, 1994). Direct measurements have indeed found that the pressure within the pre-ovulatory follicle is quite low and rupture is not a result of high intrafollicular pressure (Adashi, 1994). Mechanisms of follicular rupture are often compared to that of inflammation, as a direct result vasodilation, vascular permeability, exudation, oedema and tissue remodelling (Espey, 1994). Gene profiling of mouse COC have revealed that many genes originally believed

to be expressed exclusively in immune cells are indeed present in cumulus cells (Richards, 2007).

Considerable work has gone into understanding mechanisms of follicular rupture and many mouse models have shown that impairment of important genes can alter this process. For example, mice null for the proteolytic enzyme ADAMTS1 (a disintegrin and metalloproteinase with thrombospondin 1 motifs) demonstrate impaired ovulation which is most likely associated with a lack of protease activity (Mittaz *et al.*, 2004; Shindo *et al.*, 2000). A role for prostaglandins during follicular rupture also appears most likely. In a variety of mammalian species, the prostaglandin synthetic enzyme cyclooxygenase (COX) isoform 2 expression is up-regulated around the time of the pre-ovulatory surge. Evidence exists to suggest that prostaglandins play an important role during follicular rupture as monkeys administered with the COX inhibitor, indomethacin, into their follicular fluid experience inhibition of follicular rupture (Duffy and Stouffer, 2002). These findings are in parallel with a human study which suggests women on non-steroidal anti-inflammatory drugs experience luteinised unruptured follicle syndrome (Mendonca *et al.*, 2000), clearly suggesting that prostaglandins play a role during follicular rupture. Indeed, akin to inflammatory processes, protease activity is also associated with the process of follicular rupture. Evidence of MMPs and their inhibitors suggests that as follicles approach ovulation there is an increase protease activity (Hagglund *et al.*, 1999; Liu *et al.*, 1998). As a result of the thinning follicular wall, the epithelial cells slough off, follicle ruptures occurs and some 26-40 hours after the pre-ovulatory LH surge the COC is extruded, the follicular wall collapses and the resulting cells give rise to the corpus luteum.

1.1.5.3 Luteinisation

In concert with the release of the oocyte-cumulus complex, the LH ovulatory surge also induces luteinisation of the resulting steroidogenic cells of the dominant follicle. Prior to the LH surge, the granulosa cells of the follicle are limited to aromatisation of androgens from adjacent theca cells as they do not possess the enzymes necessary for androgen synthesis (Hillier *et al.*, 1994). However, FSH-induced maturation of granulosa cells coupled with the LH surge induces the expression of steroidogenic enzymes P450 cholesterol side-chain cleavage ($P450_{scc}$) and 3βHSD that are required for the secretion of steroid hormones by the corpus luteum.

Luteinisation can be mimicked by *in vitro* conditions. Indeed, granulosa cells will spontaneously luteinise and produce progesterone when removed from the follicular environment, even in the absence of LH (Hillier *et al.*, 1981). Therefore it appears that the follicular environment has an inhibitory effect upon the granulosa cells that can provide a milieu to discourage luteinisation (Channing *et al.*, 1980; Eppig *et al.*, 1997; Murphy, 2000). Inhibitor(s) of such magnitude have been reported to be present in follicular fluid (Channing *et al.*, 1978; Ledwitz-Rigby *et al.*, 1977) or to come directly from the oocyte itself (Brankin *et al.*, 2003; Vanderhyden and Macdonald, 1998). Therefore, it appears that the LH surge is able to remove such inhibitory factors, disrupt connections between granulosa cells and the oocyte (for example, connexins) and/or induce genes that facilitate luteinisation to transform these cells into the most steroidogenically active cells in the body.

1.2 The corpus luteum

In the first detailed description of corpora lutea (1672), Reygneir de Graaf described "globular bodies" in the ovary that formed in the place of ova and were associated with the presence of a fetus in the uterus. We now know that the transformation of a dominant follicle into the corpus luteum is one of the fundamental processes in reproductive biology.

The corpus luteum has the important role of being the interface between menstruation (non-conception cycle) and pregnancy (conception). In a non-conception cycle the corpus luteum will experience a high rate of cell turnover, intense remodelling and structural and functional demise within a 14-day period, succinctly followed by the initiation of a new cycle. Alternatively, during maternal recognition of pregnancy, the corpus luteum will continue to secrete vast quantities of steroid hormones under the trophic stimulation of conceptus- derived hCG, subsequently maintaining structural and functional integrity.

High steroidogenic output from the corpus luteum results in a reduction in ovarian cyclicity during the luteal phase. As a consequence, follicles fail to develop beyond an antral stage until luteal tissue has regressed (Zeleznik and Pohl, 2006). Such extremely

high steroidogenic output of progesterone, oestrogen and inhibin A from the active corpus luteum, are thought to exert negative feedback on the hypothalamic-pituitary axis by suppressing the trophic support of FSH and LH that is integral for pre-ovulatory follicle development (see section 1.1.2.1). Elegant studies have clearly demonstrated that removal of the corpus luteum before the expected time of luteal regression results in the prompt resumption of pre-ovulatory follicular development and ovulation approximately 14 days later (Baird *et al.*, 1984; Nilsson *et al.*, 1982). Such resumption of cyclicity is a common feature of most mammalian species and in the absence of conception it is important for the corpus luteum to be removed so that a new cycle can begin. This is clearly evident in infra-primate species such as the laboratory rodent which do not have long luteal phases and indeed require coital stimulation to produce fully functional secretory corpora lutea. This mechanism allows high levels of fecundity as ovulation and potential conception could occur every 4-5 days.

Unlike follicular development, corpora lutea development, function and subsequent demise differ greatly between species. This makes large mono-ovulatory species (cows, sheep) and the more manipulative poly-ovulatory species (rodents) very useful models of human follicular function and dysfunction in the laboratory. However, in regards to corpora lutea physiology, different functional mechanisms exist between species. Whilst these differences in reproductive patterns are meaningful in terms of adaptation of species to reproduce more efficiently under different environment and life patterns, comparative biology of corpora lutea becomes increasingly complex. On the other hand, some aspects of corpora lutea formation are similar between species for example corpora lutea formation, angiogenesis and steroid synthesis. Therefore a careful understanding of interspecies differences is integral when studying laboratory models in the context of human corpora lutea function. The next few sections will consider aspects of corpora lutea biology in regards to the focus of this thesis, the human corpus luteum, however some comparative observations will be made.

1.2.1 Formation and function of the corpus luteum

The transformation of the post-ovulatory follicle into the corpus luteum is of fundamental importance. In primates, both ovulation and corpora lutea formation are under gonadotrophic control. After follicular rupture and expulsion of the ovum, extensive

tissue and vascular remodelling is associated with the breakdown of the basement membrane of the follicle wall that once separated the avascular granulosa cells of the follicle from the theca interna layer (Luck and Zhao, 1995). The theca cell layer begins to engorge with blood, becoming oedematous and invaginating between the granulosa cell layers. This results in the follicle wall collapsing into folds characteristic of the corpus luteum (Figure 1.7). Invasion of capillaries is evident and this most likely under the influence of angiogenic and mitogenic factors (McCracken *et al.*, 1999). Although the control of corpora lutea function differs between speices, many key studies investigating luteinisation and corpora lutea development have been generated from rodent models. No evidence exists to suggest that these processes differ in the human (Behrman *et al.*, 1993), and therefore these studies remain useful in the context of human corpora lutea function.

1.2.2 Cellular component of the corpus luteum

The corpus luteum is composed of many cells types making it a heterogenous tissue in nature. This is evident in Figure 1.7 whereby the different compartments of the tissue can be morphologically identified. Steroidogenic cells exist in two forms and are often referred to as large and small luteal cells. Granulosa-lutein cells (large luteal cells) are of granulosa cell origin and make up the bulk of the volume of the corpus luteum existing in roughly a 2:1 ratio to the smaller theca-lutein cells. Both granulosa and thecal cells undergo massive hypertrophy during luteinisation, with the granulosa cells experiencing an eight-fold volume increase relative to their pre-ovulatory size (Murphy, 2000) to become the largest steroidogenic cell in the body (Fawcett *et al.*, 1969). Unlike many other species, the granulosa and theca cells of the human corpus luteum do not intermix and remain in separate clusters (Niswender and Nett, 1994) which is clearly evident in Figure 1.7. Surprisingly however, the human corpus luteum contains more non-luteal cells than luteal cells (Behrman *et al.*, 1993). Endothelial cells for example make up a substantial contribution of the cellular composition of corpus luteum. These cells are located at blood vessels and capillaries and are the result of the intense angiogenesis process that occurred post-luteinisation. Additionally, immune cells such as macrophages are well characterised to the corpus luteum, and indeed have been shown to markedly increase in number from the early to the late-luteal phase (Duncan *et al.*, 1998b). Structurally supporting cells such as fibroblasts and other components of the extracellular

matrix (ECM) are also important and prominent cells type within the corpus luteum. Fibroblasts are reported to make up a high proportion of the cells in the corpus luteum (Behrman *et al.*, 1993) and greatly increase in number during the late-luteal phase (Lei *et al.*, 1991). This may suggest that both macrophages and fibroblast cell types play integral roles during luteolysis.

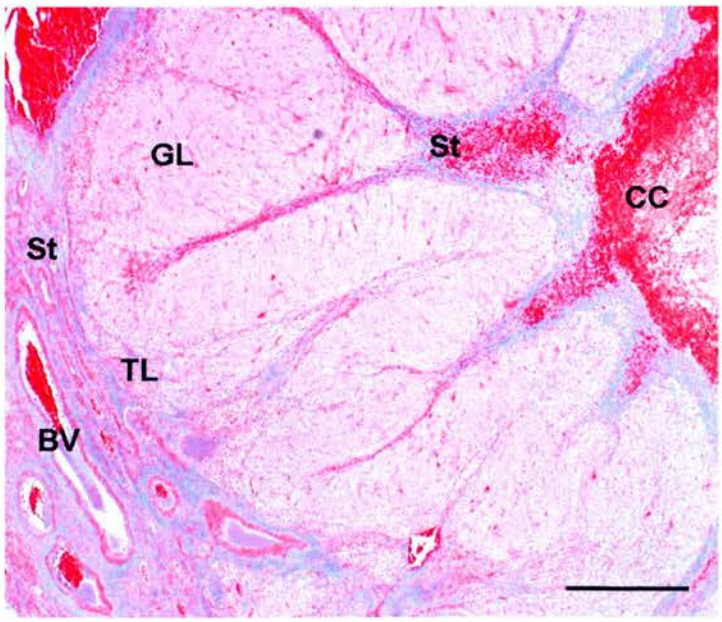


Figure 1.7 Masson's Trichrome stain of a human corpus luteum demonstrating the histological structure of the steroidogenic gland. The central core (CC) of fibrin clot and extravasated red blood cells once formed the antral cavity of the dominant follicle. Surrounding the central clot are the folds of granulosa-lutein cells (GL) that comprise the bulk of the corpus luteum. Smaller theca-lutein cells (TL), in separate clumps along with fibrous connective tissue (blue stain) invaginate between the granulosa-lutein cells. A stromal network of tissue (St) surrounds the steroidogenic cells to provide a structural network which comprises blood vessels (BV), fibroblasts and extracellular matrix. Scale bar=200 μm

1.3 Luteal steroidogenesis

The human corpus luteum is a remarkable steroidogenic gland that can produce 25-40 mg of progesterone daily (Lippsett, 1978). In women the corpus luteum also secretes vast quantities of androgens and oestrogens (Devoto *et al.*, 2002) in addition to progesterone. Hormone production in the corpus luteum is largely dependent upon the pituitary-derived LH utilising a cAMP second messenger system similar to that described in section 1.1.3.

1.3.1 Progesterone

Simultaneously crystallised and characterised by four independent groups, the discovery of progesterone as a secreted 'luteal factor' came to light in experiments involving pregnant rabbits. In these experiments the pregnant rabbits were subjected to either the removal of their ovaries or treatment with luteal extracts which resulted in abortion/resorption or survival of embryos respectively (Niswender *et al.*, 2000). This luteal extract factor became known as progesterone and was found to be the support hormone of pregnancy.

Unlike follicular granulosa cells, whereby progesterone is not synthesised, the granulosa-lutein cells of the corpus luteum have recently acquired, as a result of the pre-ovulatory LH surge, the steroidogenic machinery to generate their own progesterone. This, coupled with the newly formed vasculature provides the gland with its major source of circulating cholesterol, and the corpus luteum now has the potential to secrete vast amounts of progesterone. Although cholesterol can be synthesised de novo by the corpus luteum, it is mainly obtained from circulating plasma lipoproteins. High- and low- density lipoproteins (HDL and LDL) provide the corpus luteum with a cholesterol supply with LDL the predominant source in the human ovary (Soto *et al.*, 1984). The uptake of LDL by luteal cells is quite an efficient process. It occurs by receptor mediated endocytosis, with each LDL molecule containing roughly 2500 cholesterol molecules (Brown and Goldstein, 1986). Due to its hydrophobic nature, cholesterol cannot freely diffuse within the luteal cell and consequently requires sterol carrier proteins (SCP) to transport it from the cytosol to the mitochondria. SCP facilitate the transport of cholesterol to the outer mitochondrial membrane before being transferred to the inner mitochondrial membrane via the aqueous intermembrane space. This step involves several proteins including StAR (steroidogenic acute regulatory protein), the protein that is reported to be the rate limiting step in the steroidogenic pathway (Stevens *et al.*, 1993). Once transported into the matrix of the mitochondria, $P450_{scc}$ cleaves the side chain of cholesterol to form pregnenolone. Pregnenolone can then be transported to the closely associated smooth endoplasmic reticulum where the enzyme 3βHSD converts it to progesterone (Figure 1.8).

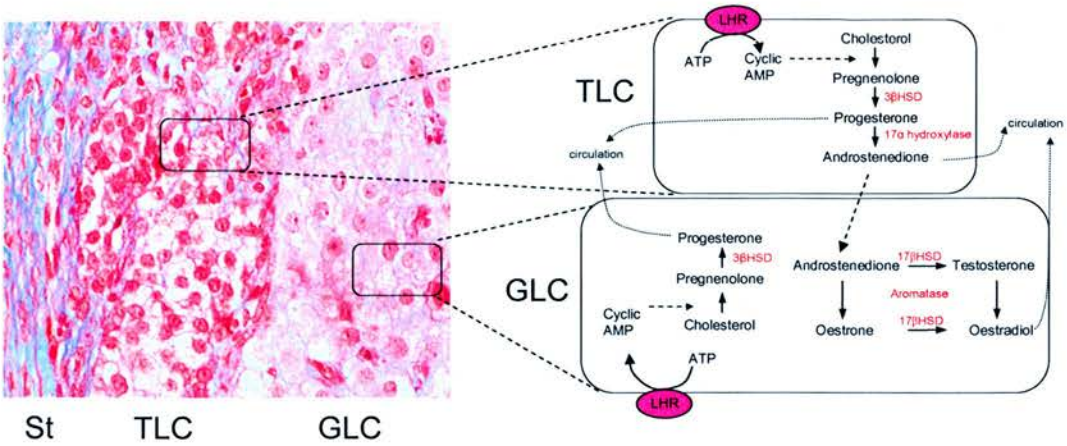


Figure 1.8 Steroidogenesis in the corpus luteum. The corpus luteum has the capacity to produce androgen, oestrogen and progesterone. The pre-ovulatory LH surge induces enzymatic changes such that the newly differentiated granulosa-lutein cells (GLC) can continue to secrete oestrogen however they can now also synthesise large quantities of progesterone due to the acquisition of 3βHSD. The adjacent theca-lutein cell (TLC) layer is no longer separated by a basement membrane and also contributes to the progesterone output. The surrounding stromal layer (St) is evident in blue which stains positively for collagen.

In all mammalian species, progesterone is the common secretory product of the corpus luteum. In a cycle whereby conception occurs and successful implantation is achieved, a viable corpus luteum is essential to ensure progesterone secretion is maintained. The principle targets of progesterone are the reproductive tract and the hypothalamo-pituitary axis. Well characterised for its role in the female reproductive tract, progesterone has the important function of preparing the uterine environment for the initiation and maintenance of pregnancy and suppression of the maternal immune response to fetal antigens (Siiteri *et al.*, 1977; Siiteri and Stites, 1982). In order for progesterone actions to be mediated in the reproductive tract, exposure to oestrogens is a pre-requisite for progesterone receptor induction (Ing and Tornesi, 1997; Kraus and Katzenellenbogen, 1993). Nuclear progesterone receptors are activated upon ligand interaction and can modulate expression of genes by binding specific response elements on the deoxyribonucleic acid (DNA).

1.3.2 Other steroidal products

In addition to progesterone, the corpus luteum is also capable of producing androgens and oestrogens (Retamales *et al.*, 1994) (Figure 1.8). The exact role of oestrogen remains questionable. For example, in some species oestradiol is known to be a luteotrophic factor (ie rat, rabbit, pig), however the dependence of the corpus luteum on oestradiol for support varies within these species (Niswender *et al.*, 2000). In humans however, addition of exogenous oestradiol has been shown to diminish progesterone synthesis by inhibition of 3βHSD in dispersed cultures of luteal cells (Vega *et al.*, 1994). Therefore it is possible that oestrogens have the opposite effect in the human corpus luteum and have luteolytic properties. Further interventional studies are required to decipher a role for oestrogen in the human corpus luteum. Regardless of the role of their role, oestradiol receptors (ER) are indeed expressed in corpora lutea of women and primates, most notably in the form of ERβ (Duffy *et al.*, 2000; Saunders *et al.*, 2000). In contrast to the follicular granulosa cells, the corpus luteum does not appear to express the ERα isoform (Chandrasekher *et al.*, 1994; Iwai *et al.*, 1990). Consistent with this observation, transgenic mice null for each receptor isoform have clearly indicated that ERβ rather than ERα has a more predominant role in the corpus luteum (Couse *et al.*, 1995; Couse *et al.*, 2005). Whether this observation extends to other species remains unknown, however due to the nature of their localisation this is probably true for humans and primates. A role for oestrogens in the corpus luteum cannot be ruled out and there may even be temporal and spatial roles. This is clearly an area lacking in current research.

1.3.3 Non-steroidal regulators

It is well recognised the corpus luteum is not only the site of steroidal hormones but also has the ability to produce and/or respond to many other factors that may act as local (paracrine/autocrine) or endocrine signals (Stouffer, 2006). Prolactin, oxytocin, relaxin and prostaglandins are common hormones considered in luteal physiology. Although the role of some of these molecules in the human corpus luteum remains elusive, they have more defined roles in corpora lutea of other species. Understanding the various mechanisms of these factors may allow a more critical understanding of the human corpus luteum. However, many species have evolved various mechanisms for controlling their reproductive activities and consequently corpora lutea function that are different

from that seen in human corpora lutea. This becomes clearly evident when discussing luteotrophic or luteolytic factors.

In rodents, prolactin (PRL)-like hormones are essential luteotrophins, whereby mating during the estrous stage of the cycle stimulates the cervix and activates a neuorendocrine loop to result in the secretion of pituitary-derived prolactin. A week later however, this prolactin secretion is taken over from the pituitary by the placenta and the pituitary is no longer essential for maintenance of pregnancy (Stouffer, 2006). Consequently, the PRL system is integral for promoting luteal structure-function in rodents. One of the key mechanisms by which this system achieves this is by directly stimulating progesterone production (Stouffer, 2006). A less convincing role for prolactin however exists in human corpora lutea. Prolactin receptors have been localised to the ovary and corpus luteum (Alila *et al.*, 1987; McNeilly *et al.*, 1980), however unlike the rodent most human and other primate studies have found that exogenous prolactin does not appear to influence progesterone production in dispersed luteal cells (Stouffer *et al.*, 1980; Tan and Biggs, 1983). Whether this is a result of the differences between species or an *in vitro* versus an *in vivo* system remains unknown.

Another protein hormone known to regulate luteal lifespan is oxytocin. Unlike domestic animals (such as cows, sheep, goats and pigs) whereby evidence suggest that oxytocin plays a role in luteal regression at the end of a non-fertile cycle, there remains no concrete role for oxytocin in the primate corpus luteum. Oxytocin is indeed expressed in the human corpus luteum, albeit at much lower levels (1000-fold) (Ivell *et al.*, 1997) than found in other species, and receptor expression is also described (Einspanier *et al.*, 1994; Fuchs *et al.*, 1990). Evidence in primates suggests that the LH surge promotes the expression and production of oxytocin and its receptor in the luteinising cells of the ovulatory follicle (Einspanier *et al.*, 1997; Ivell *et al.*, 1998) whilst others report that oxytocin interrupts progesterone synthesis (Bendz, 1977; Richardson and Masson, 1985). At least two studies however report that oxytocin infused *in vivo* to the corpus luteum shortens the luteal phase in both rhesus monkeys (Auletta *et al.*, 1984a; Auletta *et al.*, 1984b) and humans (Bennegard-Eden *et al.*, 1995), suggesting that there may be a potential paracrine role for oxytocin. Similarly, a lack of research exists also for a role for relaxin in the human corpus luteum. In many species relaxin is considered to be a hormone of pregnancy and parturition (Stouffer, 2006), however in the human limited

studies failed to demonstrated a physiological role for the hormone. The protein and mRNA have indeed been localised to steroidogenic cells of the corpus luteum (Stoelk *et al.*, 1991), however, like oxytocin, these levels are much lower than detected in other species.

In non-primate species prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has clearly been shown to be a critical regulator of the corpus luteum. Sheep, cow, pig, horse, rabbit and rat corpora lutea all experience luteolysis in response to uterine $PGF_{2\alpha}$ signal. This fundamental aspect of corpora lutea biology was first reported by Loeb in 1923 who classically demonstrated that hysterectomy in the cyclic guinea pig abolished cycles and caused persistence of corpora lutea. Hysterectomy however has no effect on the length of ovarian lifespan or corpus luteum in either primates or women, clearly demonstrating the uterine-derived $PGF_{2\alpha}$ is not of importance in higher species. This does not rule out a luteolytic role for PGF2α in primates; however the source is clearly not of uterine origin. Additional monkey studies have shown that $PGF_{2\alpha}$ does have luteolytic tendencies, however the evidence in women is less marked. Some studies have reported a fall in plasma progesterone or shortening of cycles (Embrey *et al.*, 1972) whereas others report no effects (Jones and Wentz, 1972). Whilst *in vitro* studies with cultured granulosa cells in the human do display inhibition of progesterone by $PGF_{2\alpha}$ (Henderson and McNatty, 1977), further studies are essential to further dissect out a potential role for prostaglandins in the corpus luteum of women.

1.4 Luteolysis

In the absence of pregnancy, the corpus luteum has a finite lifespan of approximately 14 days and thereafter will undergo structural and functional demise, a process termed as luteolysis. Luteolysis is absolutely obligatory for the next wave of folliculogenesis. The concept of luteolysis has been well acknowledged for many decades, however many of the regulatory mechanisms involved in the loss of function and involution of the gland are incompletely understood (Davis and Rueda, 2002). Generally, luteolysis is often divided into two distinct stages, functional and structural luteolysis. Functional luteolysis refers to the increasing failure of progesterone synthesis and secretion whilst structural luteolysis refers to the involution of the gland (Behrman *et al.*, 1993). Although it is not really understood if there are temporal and endocrinological distinctions between these

stages of luteolysis in the human, it is easier to describe them as separate events. Evidence does suggest however that, like the rat, functional and structural luteolysis may be separate events in the human as small corpora lutea of previous cycles are still evident within the ovary (Behrman *et al.*, 1993). Therefore, for purpose of this literature review, the term luteolysis will be sub-categorised into functional and structural luteolysis.

1.4.1 Functional luteolysis

Functional luteolysis is most commonly associated with the cessation of progesterone steroid output. This is generally a result of the abrogation of LH action and consequently the trophic mechanisms of support are interrupted. Surprisingly however, this is not a result of LH withdrawal, as natural luteal regression is not associated with a decrease in LH secretion; rather luteal responsiveness to LH is interrupted (Behrman *et al.*, 1993). This is also not due to LH receptor down regulation in the human at least, as mRNA expression and in situ binding studies clearly demonstrate that there are no significant changes in receptor expression throughout the lifespan of corpora lutea (Duncan *et al.*, 1996a). LH-induced progesterone synthesis declines in the presence of all other members of the steroidogenic pathway such as StAR, $P450_{scc}$ and 3βHSD (Duncan *et al.*, 1999). This suggests that the LH receptor undergoes some type of desensitisation or uncoupling from second messenger systems (Segaloff and Ascoli, 1993), which would explain why increasing doses of hCG are required to maintain progesterone production during pregnancy (Duncan *et al.*, 1996a). In contrast to human corpora lutea, rat corpora lutea do however demonstrate a ligand-induced reduction in the number of LH receptors (Hoffman *et al.*, 1991). This however this may be an example of species specific differences in the regulation of corpora lutea function.

1.4.2 Structural luteolysis

Grossly characterised by a massive decrease in size and weight, structural luteolysis involves intense tissue remodelling of the corpus luteum that is associated with cell death, an influx of immune cells and increased fibrotic tissue. Subsequently structural luteolysis can be characterised by a loss and involution of the tissue (Behrman *et al.*, 1993) which goes on to form corpus albicans. After a few cycles the corpus albicans are eventually completely reabsorbed and replaced by ovarian stroma. Therefore involution of luteal

tissue involves incredible cell loss and major remodelling of the ECM. Dissolution of the corpus luteum during luteolysis has been attributed to an increase in protease activity during the late-luteal stages in many species. Furthermore, it is clearly evident that during luteolysis there is a marked increase in macrophages (Duncan *et al.*, 1998b) and fibroblasts (Lei *et al.*, 1991), both of which can secrete the proteolytic matrix metalloproteinases (Duncan, 2000).

1.4.2.1 Matrix metalloproteinases

MMPs are a family of homologous zinc-dependent endopeptidases that play central roles in many biological processes by degrading ECM and basement membrane components. Ample evidence exists to suggest MMPs have integral roles in embryogenesis, normal tissue remodelling, wound healing, cardiovascular diseases and cancer (Chakraborti *et al.*, 2003; Visse and Nagase, 2003). MMPs are highly active proteolytic enzymes which are tightly controlled at several levels. Certain signalling pathways lead to the expression of particular MMP genes and therefore the first level of control is the activation of mRNA synthesis (Nagase and Woessner, 1999). Secondly, most MMPs are secreted as inactive zymogens (proMMP) and therefore require activation of their proMMP form. Activation of proMMPs *in vivo* occurs by tissue or plasma proteinases or opportunistic bacterial proteinases (Nagase and Woessner, 1999). A third level of MMP regulation involves endogenous inhibitors, namely α-macroglobulin and tissue inhibitors of matrix-metalloproteinases (TIMPs). An example of the tight control of MMPs can be seen with MMP-2, whereby activation requires both membrane-type 1 MMP (MT1-MMP) and TIMP-2 bound to MT1-MMP (Strongin *et al.*, 1995).

1.4.2.2 Inhibitors of matrix metalloproteinases

The major endogenous regulators of MMPs are the TIMP proteins, which bind to and inhibit MMP action with a 1:1 stoichiometry. TIMPs in general are widely expressed and although not always, they are frequently regulated in co-ordination with MMPs (Hulboy *et al.*, 1997). Currently four homologous TIMPs have been identified and all demonstrate important regulatory roles not only in matrix turnover but also in cellular activities. A second line of defence against MMPs is found in the serum in the form of α-macroglobulin (Baker *et al.*, 2002). This protein serves as a substrate for some TIMPs and after cleavage a conformational change in the inhibitor occurs that can act to entrap

and covalently bind the MMP to prevent cleavage of any other matrix molecules (Woessner, 1991).

1.4.2.3 Remodelling in the corpus luteum

The role of MMPs in remodelling in the corpus luteum has been well characterised in many species including humans. To date, over 20 different MMPs have been described with the most commonly studied being MMP-1 (collagenase), MMP-2 (gelatinase A) and MMP-9 (gelatinase B). MMPs are synthesised by connective tissue cells such as fibroblasts, endothelial cells in newly formed vessels, and infiltrating cells such as macrophages and neutrophils (Hulboy *et al.*, 1997). Considerable tissue remodelling occurs in the ovary, particularly during the processes of ovulation and luteolysis – therefore much of the MMP activity occurs in parallel with important remodelling events of the corpus luteum. Key studies looking at protease action in the human corpus luteum have shown that although the TIMPs are produced in vast quantities their expression does not consistently change over the luteal phase unlike that of MMP activity which is clearly regulated (Duncan, 2000). Further supporting a role for MMP regulation across the luteal phase is the finding that MMPs and TIMPs have differential localisation in the corpus luteum and consequently an excess of TIMPs does not necessarily fully inhibit MMP activity (Duncan, 2000; Duncan *et al.*, 1998a).

1.5 Maternal recognition of pregnancy in women

In a cycle where conception occurs, the corpus luteum is rescued from luteolysis and the structural and functional integrity of the gland is maintained. Not surprisingly, mechanisms of maternal recognition of pregnancy differ between species. A common feature however, is the requirement of progesterone secretion necessary to maintain gestation in its early stages (McCracken *et al.*, 1999). In all species, progesterone is initially produced by the corpus luteum, generally in response to LH. However, the placenta also produces progesterone in some species (sheep, horse, human) and in these species the placenta will become the predominant source of progesterone. This change in the source of progesterone is known as the luteo-placental shift. In other species (goat, pig, rabbit, mouse) the corpus luteum will continue as the sole source of progesterone throughout pregnancy and as a result the initiation of labour coupled with a uterine luteolytic signal appears to be the trigger for luteal regression (McCracken *et al.*, 1999).

In the human however, the focus of this thesis, logarithmically increasing concentrations of hCG are secreted from the developing syncytiotrophoblast and will continue to progressively rise during early pregnancy until peak levels are reached at seven to nine weeks (Kletzky *et al.*, 1985). Thereafter, hCG concentrations will decline as the placenta assumes the principal role of progesterone secretion.

HCG is a member of the glycoprotein hormone family that also includes the structurally related LH, FSH and thyroid stimulating hormone (TSH). All of these glycoproteins consist of an identical α-subunit that is non-covalently bound to a β-subunit that confers their hormone specificity. Both hCG and LH are thought to share a common receptor that belongs to the family of G protein-coupled receptors (GPCRs). Although some controversy does exist on whether hCG may have its own receptor (Gromoll *et al.*, 2000), no such gene has been identified to date. Human LHβ and hCGβ demonstrate 85% homology in the first 114 amino acids, however hCGβ is unique from the other glycoprotein β subunits because of its 24-31 amino acid extension at the C-terminus. Additionally, unlike LH subunits, the subunits of hCG contain terminal sialic acid residues that are not sulphated which markedly influence the size and charge of the molecule subsequently resulting in hCG having a greater half-life and bioactivity compared to that of LH (Stouffer, 2006).

How hCG achieves luteal rescue is one of the major questions emerging regarding luteal physiology. Whilst the obvious answer is that it is maintaining physiological progesterone concentration via interactions with the LH/hCG receptor, it is also becoming increasingly evident that hCG is having marked effects on cell types that do not express this receptor (Duncan, 2000). A clear example of this is evident in relation to MMP-2, which is localised to the stromal-fibroblast areas of the human corpus luteum, yet is regulated by hCG which can only act upon the steroidogenic cells expressing their receptor (Duncan *et al.*, 1998a). Studies are now emerging with this hypothesis in mind, aiming to understand the potential paracrine interactions that may exist between the different cell types (Duncan *et al.*, 2005b). Two emerging paracrine factors in luteal biology which are discussed in this thesis are activins and glucocorticoids.

1.6 Activins and Inhibins

Originally discovered for their opposing roles in pituitary-derived FSH modulation activin, inhibin and follistatin have been in the forefront of reproductive research for the past 20 years. Named according to the role each dimeric glycoprotein had upon FSH secretion, activin and inhibin are structurally similar but functionally diverse members of the transforming growth factor -β (TGF-β) superfamily that is comprised of over 30 members (Lin *et al.*, 2003). Follistatin is structurally unrelated to either activin or inhibin but shares the antagonist-like properties of inhibin by irreversibly binding and subsequently neutralising activin actions (discussed in section 1.6.4.2). Although originally discovered in a reproductive context, the importance of the activin/inhibin/follistatin system has been extrapolated to many other body systems such as wound repair (Sulyok *et al.*, 2004), inflammation (Jones *et al.*, 2000a), fetal development (Matzuk *et al.*, 1995), cell proliferation and apoptosis (Hully *et al.*, 1994). The following paragraphs will review the nature and actions of activin, inhibin and follistatin in regards to female reproduction, namely in the ovarian follicles and corpus luteum.

1.6.1 Activin

After the initial purification of inhibin from porcine follicular fluid, another compound was discovered which could positively modulate FSH release from pituitary gonadotrophes *in vitro* (Ling *et al.*, 1986; Vale *et al.*, 1986). This compound was termed activin and is now recognised as one of the most important growth factors produced both locally and systemically in many tissue systems. Comprised of two mature inhibin β subunits, activins are dimeric proteins of approximately 25kDa. Multiple forms of activins exist due to the various β subunit isoforms, all of which are encoded by different genes. To date five isoforms of the β subunit have been cloned with βA and βB being the best characterised. Activin A (βA:βA), the major focus of this thesis, and activin B (βB:βB) are homodimers composed of two βA and two βB subunits respectively. Activin AB (βA:βB) is a heterodimeric protein consisting of both a βA and βB subunit. The homology between the βA and βB subunits is approximately 63% (Chang *et al.*, 2001) and the β subunit is highly conserved between species (mature βA is 100% conserved between human, cow, rat and sheep; whilst mature βB differs by only one amino acid).

This suggests important function and evolutionary pressure to maintain the molecular integrity of inhibin family members (Vale *et al.*, 1990).

The more recent discoveries involving the inhibin/activin β subunit family have been termed βC, βD and βE and have been cloned from human liver (Hotten *et al.*, 1995), Xenopus laevis (Oda *et al.*, 1995) and mouse liver (Fang *et al.*, 1996) respectively. Reproductive roles have been suggested for βC as the mRNA has been found by Loveland and colleagues (Loveland *et al.*, 1996) in the human ovary, placenta and testis. This thesis will focus on activin A and to a lesser extent activin B.

Activin A is the major activin found in the circulation whilst activin B is reported to be predominantly produced in the pituitary. A pituitary-ovarian axis model proposed by Muttukrishna and colleges aims to demonstrate disparate roles of activin A and activin B within the hypothalamic-pituitary gonadal (HPG) axis (Muttukrishna *et al.*, 2004). This model suggests that activin A acts more as an endocrine modulator of pituitary FSH whilst activin B has more of an autocrine/paracrine role for pituitary derived FSH secretion. The major source and principal site of βA, βB and follistatin expression in the ovary are the follicular granulosa cells and the granulosa-lutein cells.

1.6.1.1 Extragonadal activin A

Extragonadal sources of activin A have been identified in non-ovarian sites including bone marrow, human leukemia cells (Lockwood *et al.*, 1996) and immune cells (Phillips *et al.*, 2005). When looking at the serum profiles of activin A, inhibin A, FSH/LH, progesterone and oestradiol, it is evident that activin A is negatively correlated with the other hormone profiles (Muttukrishna *et al.*, 1996) (Figure 1.9). Whilst inhibin A and steroidal hormones fall at the end of the luteal phase, activin A however is maximal, suggestive that it may have a positive role in luteolysis. This source of activin A in the circulation is probably not from the follicles as there is no difference in serum activin A concentrations between women undergoing gonadotrophin-induced follicular development compared to those in non-stimulated cycles (Knight *et al.*, 1996). Coincident with the rise in serum activin A during the late-luteal phase, the corpus luteum maybe a possible source of the rising protein concentration or alternatively may just be a paracrine target of its actions.

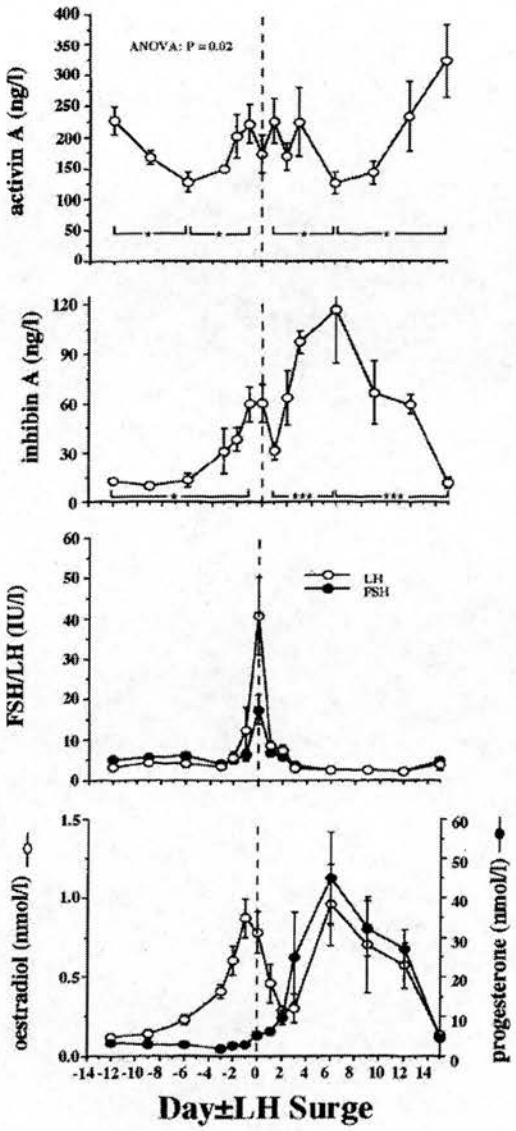


Figure 1.9 Mean serum profiles of activin A, inhibin A, the gonadotrophins and steroid hormones over the ovarian cycle of five spontaneously cycling women. Unlike inhibin A, FSH, LH, oestradiol or progesterone, activin A is maximal in the late-luteal phase suggestive that it may play an important role in the luteolytic mechanism.

(Muttukrishna *et al.*, 1996)

1.6.2 Inhibin

Although first described in the seminal plasma of normal men, the heterodimeric protein inhibin was first isolated and purified in bovine follicular fluid by scientists in Melbourne, Australia (Robertson *et al.*, 1985). The mature form of the inhibin protein is approximately 32 kDa and is composed of an α subunit and β subunit as described above. The two isoforms of inhibin that have been isolated from follicular fluid are inhibin A (α:βA) and inhibin B (α:βB), which share the same α subunit but like the different activin isoforms are encoded by different β subunit genes.

1.6.3 Activin receptors and intracellular signalling

Activins elicit their responses via extracellular reception and an intracellular signalling system. The activin receptors were cloned and characterised in the early 1990s (Zimmerman and Mathews, 1996) and like all members of the TGF-β superfamily use a common mechanism to signal to the nucleus. Activin signalling events are initiated in response to activin binding to its two types of transmembrane serine/threonine receptor kinases, activin type II receptors (ActRII, ActRIIB) and activin type I receptor (activin receptor-like kinase; ALK). Upon ligand binding, two of each receptor type are brought together in order to form an activated stable receptor complex. Activin type II receptors are the primary ligand binding proteins, and even in the absence of type I receptors they have a very high affinity for the activin protein (Mathews and Vale, 1991). Once in the activated receptor complex, the binding of the activin ligand results in the constitutively active type II receptor phosphorylating the type I receptor and subsequently activating the kinase domain (Attisano *et al.*, 1996). Once activated, ALK4 can then phosphorylate the cytoplasmic intracellular Smad (named after the sma genes and the human homologue of Drosophila Mothers against decapentaplegic) signalling proteins which initiate a signalling cascade that ultimately regulates gene transcription and expression.

The Smad proteins are a group of evolutionarily conserved proteins that act as mediators of transcriptional activation by members of the TGF-β superfamily, including activins, bone morphogenic proteins (BMPs) and GDF9. Members of the Smad family are divided into three distinct classes. The receptor-activated Smads (R-Smads), the common-mediated Smad (Co-Smad) and the inhibitory Smads (I-Smads). Essentially, once the R-Smad (Smad 2 or 3 for the TGF-β/activin signalling pathway or Smad 1, 5 or 8 for the

BMP pathway) has been phosphorylated by ALK4 it forms a complex with the co-Smad (Smad 4, common to both TGF-β/activin and BMP signalling pathways) which is then free to translocate from the cytoplasm into the nucleus to regulate target genes by DNA binding and recruitment of transcriptional co-factors (Welt *et al.*, 2002) (Figure 1.10). Increased expression of the I-Smads (Smad 6 for BMP signalling and Smad 7 for TGF-β/activin signalling pathway) however can inhibit activin signalling by two known inhibitory mechanisms. To negatively regulate the signalling pathway, I-Smads can form stable complexes with ALK4 to inhibit pathway-specific Smad phosphorylation and/or they can compete with Smad 4 for binding to phosphorylated pathway-specific Smads, preventing formation of transcriptionally active Smad complexes (Zimmerman and Mathews, 2001). In addition to the I-Smads there are other intracellular modulators of activin signalling, for example a protein named SARA (Smad anchor for receptor activation) (Welt *et al.*, 2002). SARA is capable of binding to unphosphorylated Smad 2 and modulating its access to type I activin receptor, thereby masking its nuclear localisation signal and maintaining it in a cytoplamic distribution (Welt *et al.*, 2002). Conversely, upon receptor activation, SARA can also interact with the type I activin receptor and facilitate Smad 2 phosphorylation, dissociate from the Smad, allowing it to form a complex with Smad 4 and translocate into the nucleus.

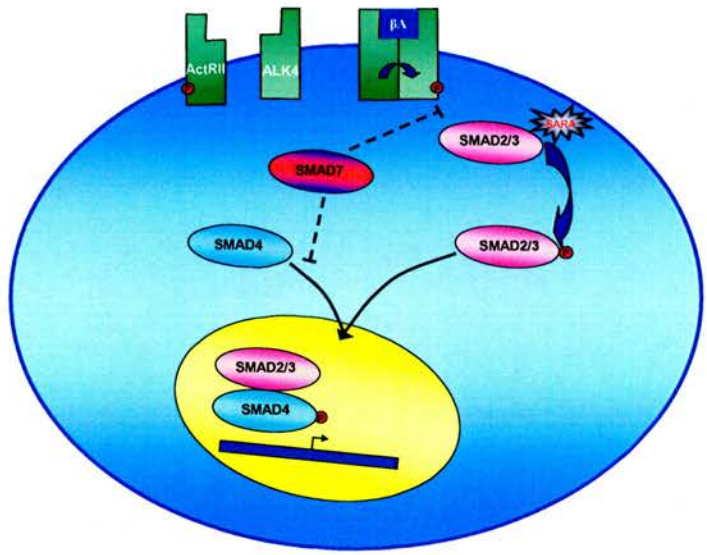


Figure 1.10 Schematic diagram of the activin receptor and intracellular Smad signalling proteins. In the presence of an activin ligand activin receptor type II (ActRII) will recruit and phosphorylate activin type I receptor (ALK4) forming an actively stable receptor complex. ALK4 with the aid of SARA which stablises the complex, activates the SMAD 2/3 protein in the cytoplasm (blue) which is then able form a complex with the common SMAD 4. This complex then translocates into the nucleus (yellow) to regulate target genes. The inhibitory SMAD 7 can antagonise these responses primarily by associating with the receptors and preventing SMAD 2/3 activation.

1.6.4 Inhibitors of activin action

One of the unique characteristics about activin and inhibin is that although they are structurally very similar, functionally they are diverse. Although activin acts on more cell types than inhibin, in cells where both proteins have biological effects, their actions are always antagonistic (Vale *et al.*, 1990). The proposed principal mechanism of action by which inhibin exerts its antagonistic effects upon activin action is by interfering with activin signal transduction. The next section describes the role of follistatin, inhibin and the inhibin co-receptor β-glycan in mediating functional antagonism of activin signalling.

1.6.4.1 Follistatin

Unlike activin or inhibin, follistatin is a monomeric protein that like inhibin, was initially discovered for its ability to inhibit FSH activity. It was not until the late 1980s (DePaolo *et al.*, 1991) however until the physiological significance of follistatin was identified. It became evident that follistatin was a high-affinity activin-binding protein that could modulate the biological availability of activin at the target cell level (Kogawa *et al.*, 1991; Nakamura *et al.*, 1990). Although follistatin binds to both activin and inhibin via their common β subunits (two follistatin molecules for each activin β/β homodimer) it is believed that only activin action is neutralised by the interaction (Shimonaka *et al.*, 1991). The dissociation rate of the activin-follistatin complex is so slow that the interaction between the two proteins is rendered irreversible (Schneyer *et al.*, 1994). As a result follistatin inhibits activin by preventing it from binding to its own receptors.

Follistatin is encoded by a single gene, however alternative splicing of the mRNA gives rise to two different precursor forms; follistatin 344 and follistatin 317 (Shimasaki *et al.*, 1988). From the two precursor forms, removal of the signal peptide results in mature peptides of 315 and 288 amino acids respectively (Figure 1.11). Further forms of follistatin can be cleaved at the carboxy-terminus by proteolytic enzymes, however the two major forms, follistatin 315 and follistatin 288 will be discussed. Both forms of follistatin are able to neutralise activin activity although it has been reported that follistatin 288 has a higher affinity for activin than the 315-residue molecule (Hashimoto *et al.*, 2000). It is generally thought the follistatin 315 is the predominant follistatin in the circulation (Schneyer *et al.*, 1996), although measurable quantities of follistatin 288 are detected in the serum (de Kretser *et al.*, 2002; Evans *et al.*, 1998). Most of the follistatin in the circulation is bound, presumably to activin, although it can bind to other molecules such as α2-macroglobin and factors with structural homology such as inhibin, BMP-4 and BMP-7 (Phillips *et al.*, 2001). This suggests that follistatin most likely acts as neutralising agent and does not have any endocrine actions of its own (Khoury *et al.*, 1995).

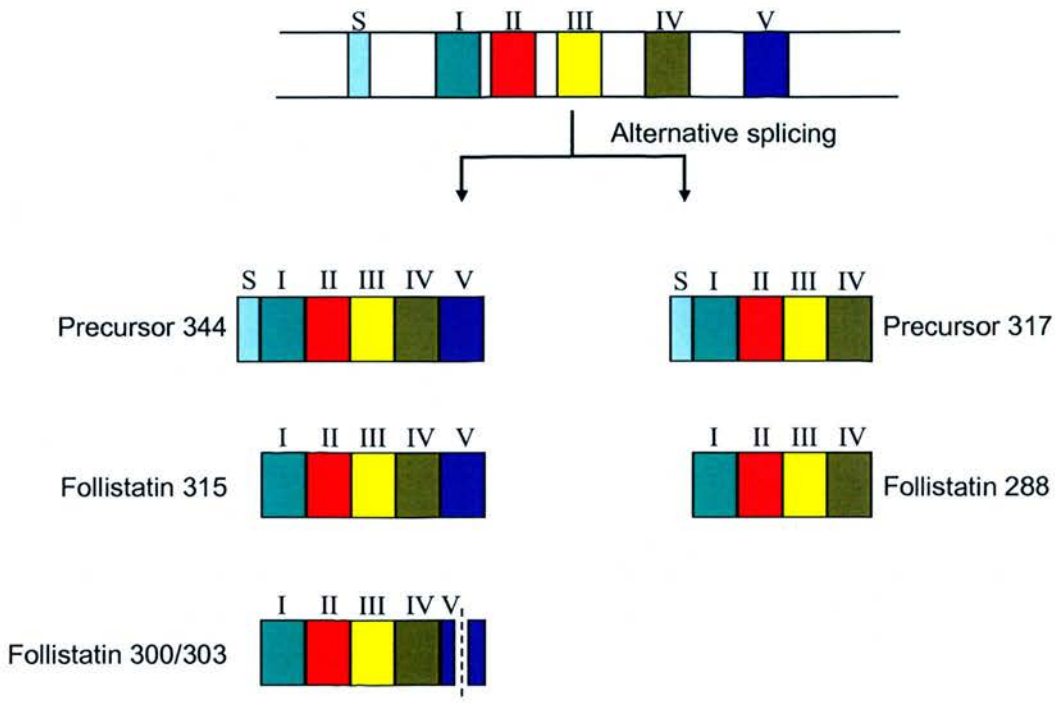


Figure 1.11 Schematic representation of the follistatin gene and the alternate splicing of the six exons that generates two major forms of pre-proteins (344 and 317). Removal of the signal peptide (S) leaves mature peptides of follistatin 315 and follistatin 288, whilst the 315 form can be further cleaved by proteolytic enzymes (dashed line on exon 5) to form a truncated form of follistatin 315 (approx. 300 amino acids) which is particularly abundant in follicular fluid.

Adapted from (Phillips *et al.*, 2001)

1.6.4.2 Inhibin and β-glycan

So far, activin actions with two types of transmembrane serine/threonine receptor kinases have been discussed. Although structurally related, the functional properties of inhibin are very diverse in comparison to that of activin. Because of the structural properties of inhibin can also bind to the type II activin receptors (particularly type IIB) via the common β subunit, with up to 40% competition for activin-binding (Lebrun and Vale, 1997; Robertson *et al.*, 2000). However, unlike activin binding, the interaction of inhibin and the activin type II receptor does not result in recruitment of the ALK receptor and therefore signalling via the Smad cascade does not occur. This is thought to be due to the lack of a second β subunit and although the α subunit is similar, it is unable to recruit the ALK receptor and form a stable signalling complex (Robertson *et al.*, 2000; Xu *et al.*,

1995). As a result, inhibin binding to the activin receptor type II exerts antagonistic actions as it prevents activin binding its own receptor. Therefore inhibin impedes activin action at the level of the receptor.

The quest for an inhibin receptor has been an on going enigma for many research groups, coupled with the fact that inhibins fail to antagonise activin in some tissue and cells, it appears that additional components may well be required for inhibin action (Draper *et al.*, 1998; Hertan *et al.*, 1999; Lebrun and Vale, 1997). Elegant studies in the Vale laboratory at the Salk Institute have shown that the TGF-β III receptor β-glycan, can function as an inhibin co-receptor with ActRII (Lewis *et al.*, 2000). These studies clearly demonstrate that β-glycan can function as an inhibin co-receptor by binding with high affinity to the protein and enhancing the interaction in cell that co-express ActRII and β-glycan (Lewis *et al.*, 2000). Indeed, protein localisation studies using anti β-glycan antibodies have shown positive staining in the oocyte, follicular granulosa and thecal cells and steroidogenic cells of the corpus luteum (Drummond *et al.*, 2002; Lewis *et al.*, 2000; Liu *et al.*, 2003), all cells which have been reported to express activin receptors. It should be noted that studies using rat tissue (Drummond *et al.*, 2002; Lewis *et al.*, 2000) described positive staining in all stages of follicular development, whilst the human study (Liu *et al.*, 2003) reported primordial, primary, pre-antral and early antral follicles β-glycan negative. Therefore in human ovarian tissue protein localisation of β-glycan is predominant in the later follicle stages that coincide with activin inhibition or down-regulation (Figure 1.12). Presumably this coincides with the developmental stages when inhibin is also maximal and the sensitivity of inhibin binding to the activin receptors is increased in order to impede activin action.

1.6.4.3 α-2 macroglobulin

Another extra-cellular binding protein with an affinity for activin is the plasma proteinase inhibitor α2-macroglobulin (Niemuller *et al.*, 1995). Like that of follistatin, α2-macroglobulin can bind to activin and neutralise its bioactivity, however it does this with a lower affinity than that of follistatin (Niemuller *et al.*, 1995). As α2-macroglobulin is found in the extracellular spaces and circulation in large quantities it has been proposed to act as a high abundance, low-affinity binding partner for activin A (Phillips, 2000).

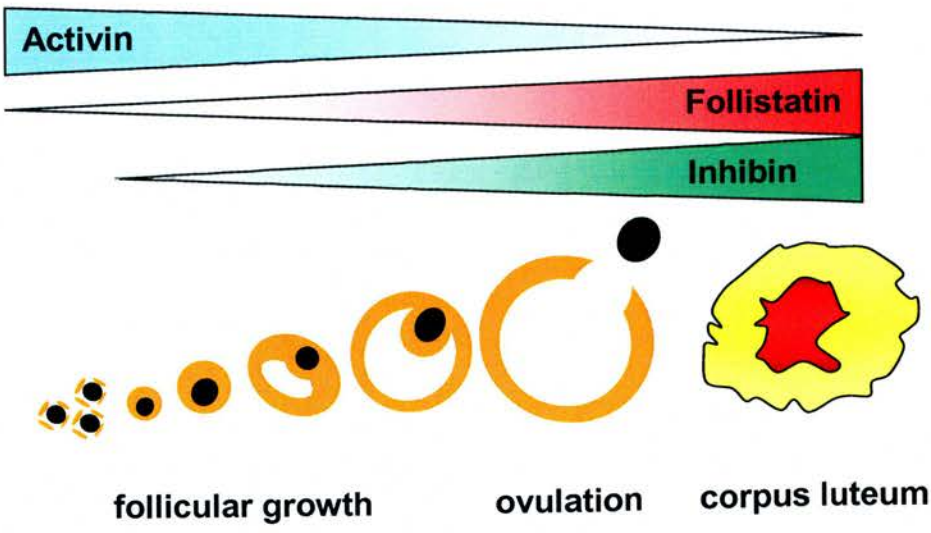


Figure 1.12 The likely developmental-related changes of activin, inhibin and follistatin in the human ovary. Collective studies suggest that smaller follicles are exposed to and reliant on activin, however once the follicles acquire gonadotrophin responsiveness it appears that activin action is down-regulated due to an increase in inhibitors such as follistatin and the structurally related inhibin protein. This phenomenon appears to be important as the dominant follicle undergoes the folliculo-luteal transition into the corpus luteum, until in the late-luteal phase when activin levels begin to rise again.

Adapted from (Knight and Glister, 2001)

Although initially characterised for their ability to regulate FSH secretion, activins are have been found to have a diverse range of functions and effects. They have been characterised in a plethora of physiological systems and found to play integral roles in many processes ranging from fetal development to the innate immune responses to reproductive function. Within the ovary, activins are important regulatory molecules that have a wide range of effects within female reproduction. Activins are also tightly regulated at many levels. Inhibitors of activin action are present in the ovary along with activin receptors and the signalling components of the intracellular Smad pathway. This review of the literature has focused upon activins and considered the sex steroids progesterone, testosterone and oestrogen; however another steroid worth consideration are the glucocorticoids.

1.7 Glucocorticoid synthesis and regulation

Synthesised in the zona fasciculata of the adrenal cortex in response to adrenocorticotrophic hormone (ACTH), glucocorticoids are known to exert diverse actions throughout the body. Produced from a cholesterol precursor in the same manner as described in the 'two-cell, two-gonadotrophin' model of oestrogen synthesis (Figure 1.5), progesterone and 17α hydroxyprogesterone are converted to mineralocorticoids and glucocorticoids via the 21hydroxylase and 11βhydroxylase enzymes (Figure 1.13). Most studies confer that there is no 21hydroxylase enzyme in the ovary, however a recent study reports for the first time that this enzyme is indeed localised to the luteinised granulosa cells from macaque and increases following an ovulatory synthesis (Fru *et al.*, 2006).

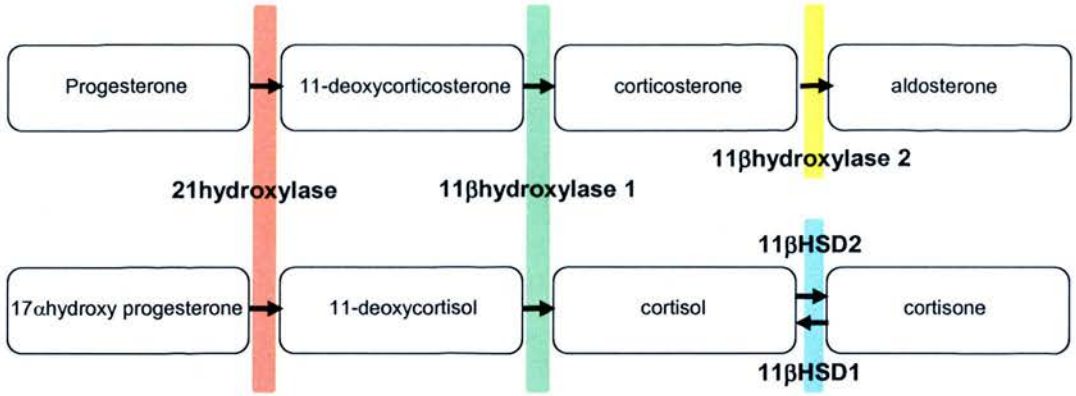


Figure 1.13 A model of mineralocorticoid (aldosterone) and glucocorticoid (cortisone/cortisol) synthesis. Progesterone and its metabolite 17α hydroxy progesterone are catalysed to 11-deoxycorticosterone and 11-deoxycortisol respectively by 21hydroxylase. The enzyme 11βhydroxylase1 is responsible for the conversion to corticosterone and cortisol whilst the 11βHSD enzymes regulate metabolism between cortisol and cortisone.

Glucocorticoids (cortisol and cortisone) have been documented to have an array of physiological roles including regulation of carbohydrate and amino acid metabolism, maintenance of blood pressure, and modulation of stress and inflammatory responses (Munck and Naray-Fejes-Toth, 1992; Tomlinson *et al.*, 2004). Cortisol is the predominant circulating glucocorticoid in humans and is tightly controlled at various molecular levels. The majority of circulating cortisol (more than 90%) is bound to a cortisol binding protein (CBP) and other mechanisms such as 11βHSD2 enzyme exist in

order to protect both the mineralocorticoid and glucocorticoid receptor (GR) from excessive active cortisol.

1.7.1 11β hydroxysteroid dehydrogenase enzyme

At the tissue level, the 11βHSD isoforms act to regulate the local bioavailability of glucocorticoids. The 11βHSD isoforms, namely 11βHSD1 and 11βHSD2 are biochemically distinct isoforms encoded by separate genes and share little homology (<15%) in their primary sequence (Albiston *et al.*, 1994; Michael *et al.*, 2003). Both isoforms are members of the short chain alcohol dehydrogenase superfamily that also include other mammalian and bacterial HSD enzymes (Michael *et al.*, 2003). Type 1 11βHSD functions as either has a dimer or tetramer to predominantly convert inactive cortisone, by nicotinamide-adenine dinucleotide phosphate (NADPH)-dependent reduction, into the active form of cortisol (Figure 1.14). Although 11βHSD1 is bidirectional and can subsequently inactivate glucocorticoids, this direction of conversion is dependent upon the NADPH state of the cell, which in steroidogenic tissue such as the ovary and testis is reported to favour 11βHSD1 activity (Michael *et al.*, 2003). Type 2 11βHSD however is a nicotinamide-adenine dinucleotide (NAD+) dependent unidirectional enzyme that works to inactivate glucocorticoids (Albiston *et al.*, 1994). This shuttle system of glucocorticoid activation/inactivation operates to control the access of active glucocorticoids to GR.

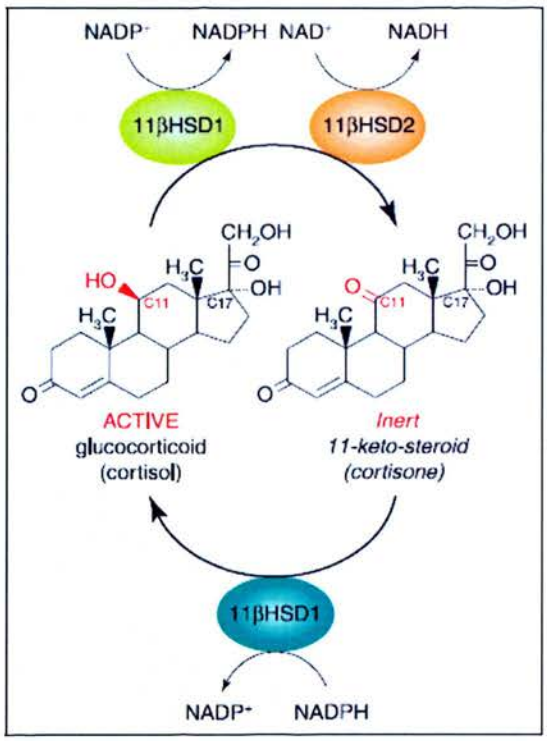


Figure 1.14 The 11βHSD enzymes exist in two isoforms. Interconversion of cortisol and cortisone occurs by oxidation or reduction at carbon position 11. The 11βHSD1 isoform acts predominantly as an NADP(H)-dependent reductase to generate active cortisol whilst 11βHSD2 is a high affinity NAD+ -dependent enzyme that catalyses the inactivation of glucocorticoids. This is often referred to as the glucocorticoid shuttle as these enzymes essentially catalyse glucocorticoid activation and inactivation.

(Michael *et al.*, 2003)

1.7.2 The glucocorticoid receptor

Glucocorticoids elicit their responses via GR encoded by a 10 exon gene which is ligand induced transcription factor, that is member of the nuclear receptor superfamily (Duma *et al.*, 2006). Inactive GR is located in the cell cytoplasm as a hetero-oligomeric complex containing chaperone proteins such as heat shock proteins (HSP) and others. When the glucocorticoid ligands bind, GR undergoes a conformational change such that the chaperones dissociate and nuclear localisation sequences are revealed which enable translocation of the ligand-receptor complex into the nucleus. The GR is highly conserved throughout different species (Encio and Detera-Wadleigh, 1991) due to the

profound influence of glucocorticoids upon development, differentiation and survival. This is clearly evident in mice which due to a deletion of exon 2, are null for GR and experience severe abnormalities and die within two hours of birth (Cole *et al.*, 1995).

Alternative splicing of GR gives rise to two receptors, GRα and GRβ (Hollenberg *et al.*, 1985). Most work reported on GR has involved the classical receptor, GRα. This isoform is present is almost all human tissues and cells and functions as ligand-dependent transcription factor. GRβ however is expressed in many human tissues and cell but usually at lower concentrations than GRα (Duma *et al.*, 2006). In addition, unlike GRα which as previously mentioned is present in either the cytoplasm or nucleus depending upon ligand availability, GRβ is constitutively localised to the cell nucleus (Duma *et al.*, 2006). To date, no functional role for GRβ has been identified in the ovary, however it has been localised to fetal trophoblast cells (Gupta *et al.*, 2003) suggesting that it may potentially be present in other reproductive tissues. Most work investigating a putative role for this isoform have found that GRβ acts as a dominant negative inhibitor of GRα mediated transcription and may be involved in the regulation of tissue-specific sensitivity to glucocorticoids in several pathological conditions (Duma *et al.*, 2006; Hammond, 2002; Shetterly *et al.*, 2001). Further work needs to be carried out in order to identify whether GRβ may play a role in reproductive and indeed ovarian function.

1.7.3 Glucocorticoids in the ovary

Much evidence exists to suggest that glucocorticoids have role in the ovary and indeed during corpora lutea formation. Regardless of whether the ovary itself can synthesise glucocorticoids, it can most definitely regulate the local environment via the 11βHSD enzymes. The temporal and spatial expressions of both 11βHSD isoforms have been well documented in the ovary many species (Ricketts *et al.*, 1998; Smith *et al.*, 2000; Tetsuka *et al.*, 1997; Yong *et al.*, 2000). It is well established that during the follicular phase, in follicles that have not been exposed to the pre-ovulatory LH surge that 11βHSD2 is the predominant isoform (Tetsuka *et al.*, 1997). This is an important concept as too much cortisol during the follicular phase is reported to disrupt FSH-stimulated granulosa cell development/function (and presumably oestradiol production) (Hsueh and Erickson, 1978), that would consequently inhibit successful folliculogenesis.

After the pre-ovulatory surge there appears to be a switch in 11βHSD isoform, with 11βHSD1 becoming the predominant form (Ricketts *et al.*, 1998; Smith *et al.*, 2000; Tetsuka *et al.*, 1997; Yong *et al.*, 2000). Therefore, as pointed out by Michael et al., it appears that the type of 11βHSD expression (type 1 or type 2 isoform) is dependent upon the differential state of the granulosa cell (Michael *et al.*, 2003) and results in a net result of increased cortisol production as ovulation approaches. One of most characterised roles of cortisol in reproduction is during the ovulation process and the resulting folliculo-luteal transition. This process has been likened to an inflammatory-like process as a result of the acute haemodynamic, cellular and biochemical changes that occur at the site of follicle rupture (Espey, 1980; Hillier and Tetsuka, 1998). Many key studies have clearly shown that the repetitive damage from consecutive ovulations must be quickly repaired in anticipation for the next ovulatory cycle, and locally produced glucocorticoids are indeed involved (Gubbay *et al.*, 2005; Rae *et al.*, 2004a; Yong *et al.*, 2002).

Therefore it is a well established that 11βHSD1 is the predominant isoform after the pre-ovulatory LH surge, suggesting that cortisol levels are relatively high in the developing corpus luteum. Andersen proposes in his 'free hormone hypothesis' that such high levels of cortisol may be vital to reduce or prevent unwanted inflammatory events from taking place (Andersen, 2002). Much less work however has focused upon a functional role for cortisol beyond ovulation and corpora lutea formation, looking at functional role for glucocorticoids over the functional lifespan of the corpus luteum. There is one exception however from a study which looked at the pregnant rat corpus luteum. Although governed by different luteolytic mechanisms than in women, this study elegantly described a potential role for 11βHSD2 in the regressing corpus luteum (Waddell *et al.*, 1996).

1.8 The general scope of this thesis

As discussed, the human corpus luteum is one of the most active endocrine glands in the body. Not only does it experience extensive tissue and vascular remodelling in a transient and predictable fashion, it has a steroidogenic output unparalleled to any other tissue. This thesis reports work on the human corpus luteum utilising both observational and interventional approaches. The careful collection and precise dating of human corpora lutea from women in this study provide the opportunity to map the functional and structural changes that occur over the normal luteal lifespan. Furthermore, unique to our laboratory is the 'rescued' corpora lutea, which complement the normal luteal phase data. Collection of rescued tissue from women given daily doubling doses of exogenous hCG in the mid-luteal phase allows temporal and spatial aspects of maternal recognition of pregnancy to be studied. This tissue has proved to be indeed an integral aspect of the present study.

To fully complement the observational studies which utilised human corpora lutea, an interventional model system was also used. This involved dispersed cultures of human luteal cells in two distinct forms obtained from women undergoing IVF (*in vitro* fertilization) assisted conception programmes. Steroidogenic cells in the form of luteinised granulosa cells and stromal cells in the form of fibroblast-like cells were also key tools in this thesis. This *in vitro* system allowed potential *in vivo* interactions to be modelled and further confirmed with the observational human luteal phase material.

Although many investigators work on the corpus luteum, very few of these studies utilise human models. Therefore careful interpretations of animal data are warranted when applying findings to human relevance as species differences clearly exist. Furthermore, this makes the literature sometimes less useful and applicable to the current study. This thesis investigates paracrine and endocrine influences on human luteal cells in four experimental chapters. Together, these findings employing both the *in vivo* and *in vitro* models aim to give insights into some of the functional mechanisms that control the human corpus luteum.

Fundamental to this thesis is the concept that hCG has effects upon cell types in the corpus luteum that do not express the hCG/LH receptor. Therefore paracrine signalling molecules must be involved. Chapter 3 sets the scene with the aim of investigating a role

for activin A in the tissue remodelling that is associated with human luteolysis. It became evident whilst investigating a role for activin A, the regulation of hCG upon activin A, activin inhibitors and the activin signalling pathway, that hCG had disparate effects upon other potential effector molecules. Consequently a role for the glucocorticoid cortisol was considered with the anticipation that the opposite effects upon tissue remodelling (or indeed an opposing role) to that of activin A would be evident (Chapter 4). As an emerging role for activin A was clearly evident during the luteolytic period in the corpus luteum, it became apparent that activin A may indeed be an anti-luteal molecule that would therefore have opposing effects to the luteotrophic hCG. This concept was investigated in relation to many important genes involved in luteinisation and accordingly forms the crux of Chapter 5. Finally, Chapter 6 explores a potential role for activin B in the human corpus luteum in order to decipher if this less considered glycoprotein has a paracrine role as fundamental to corpora lutea regulation as activin A appears today. Herein this thesis provides novel data that, for the first time, clearly demonstrates that activin A is an excellent candidate molecule as an important regulator of the human corpus luteum; one of the major enigmas in human reproductive biology.

2 General Materials and Methods

2.1 Collection of human material

2.1.1 Ethical Approval

The collection and use of corpora lutea used in this study was approved by the Reproductive Medicine Sub-Committee of the South East Scotland Medical Ethics Committee and reapproved by the common reproductive ethics committee (COREC) on two further occasions. Informed consent was obtained from all patients and their consultant gynaecologists. The recruitment of patients and the collection of tissue was initiated and performed by Dr W. Colin Duncan during his Doctor of Medicine under the supervision of Dr Peter Illingworth.

The Reproductive Medical Subcommittee and subsequently COREC separately approved the collection of cells from patients undergoing assisted conception at Edinburgh Fertility and Reproductive Endocrine Centre (EFREC). Informed consent was obtained from all patients and their consultant gynaecologists, notably Dr K. Joo Thong.

2.1.2 Corpora lutea

Human corpora lutea collected over the past 12 years (Duncan *et al.*, 2005b; Duncan *et al.*, 1998a) were enucleated at the time of surgery from women with regular menstrual cycles undergoing hysterectomy for benign conditions and dated on the basis of the urinary LH surge as described previously (Duncan, 2000; Duncan *et al.*, 1996b). Briefly, all the women in the study were on the waiting list for hysterectomy at the Royal Infirmary of Edinburgh and were under the age of 45 years. Healthy women, with regular menstrual cycles, who had not received any form of hormonal therapy in the last three months, who did not have a history of infertility, and who were having open abdominal hysterectomy for benign conditions, were identified.

2.1.2.1 Collection of human corpora lutea

Upon collection at operation, the corpus luteum was quartered to ensure that each quarter contained all cellular elements. Two quarters were immediately frozen and stored at

-70°C until RNA extraction was carried out. The remaining tissue was fixed in 10% neutral buffered formalin for subsequent immunohistochemistry.

2.1.2.2 Dating of human corpora lutea

Women participating in the study were asked to collect urine samples during the days leading up to their operation so that their normal hormone production could be assessed. Of all the women who collected urines, 65% had a suitable corpus luteum collected at surgery. Urine samples were used to measure LH concentrations and the LH surge was defined as the date of peak urinary LH after standardisation for creatinine concentrations. The corresponding corpus luteum was not classified into one of the defined stages (and consequently not used for definitive studies) if there was no LH peak or there was more than one disparate peak in the urinary samples.

2.1.2.3 Classification of human corpora lutea

Corpora lutea were classified into one of three defined stages. If collected one to five days after the urinary LH peak (LH+1 to LH+5), corpora lutea were classed as early-luteal; mid-luteal was days six to ten (LH+6 to LH+10); and late-luteal as days eleven to fourteen (LH+11 to LH+14). Plasma progesterone concentrations were obtained from patients and are depicted in Figure 2.1.

2.1.2.4 Addition of exogenous hCG to mimic 'luteal rescue' in patients

In order to research into the establishment of early pregnancy, a regime was set to administer exogenous hCG to women in the mid-luteal stage of their cycle. Daily intramuscular injections of hCG were given from LH+7 until operation (5-8 days) with dosage beginning at 125 IU (LH+7) and doubled daily until 16,000 IU at LH+14.

2.1.2.5 Maintenance and re-establishment of archived human corpora lutea

In the previous years (roughly 1995-1998) corpora lutea tissue has been readily available for research purposes from women undergoing surgery for benign conditions. More recently however, women are able to obtain medical and not surgical therapy for such cases, and consequently the availability of tissue and indeed corpora lutea has become scarce. As a result approximately only 4-5 corpora lutea have been collected in the duration of the present study. During this time, tissue collection has been performed by

technical staff in Prof Hamish Fraser's laboratory under the guidance/instructions of Dr W. Colin Duncan. All recently collected tissue is treated in very similar conditions to that of earlier studies (Duncan *et al.*, 1996a; Duncan *et al.*, 1996b) with the exception of a sliver of material being collected for primary cell culture experiments (see section 3.2.3).

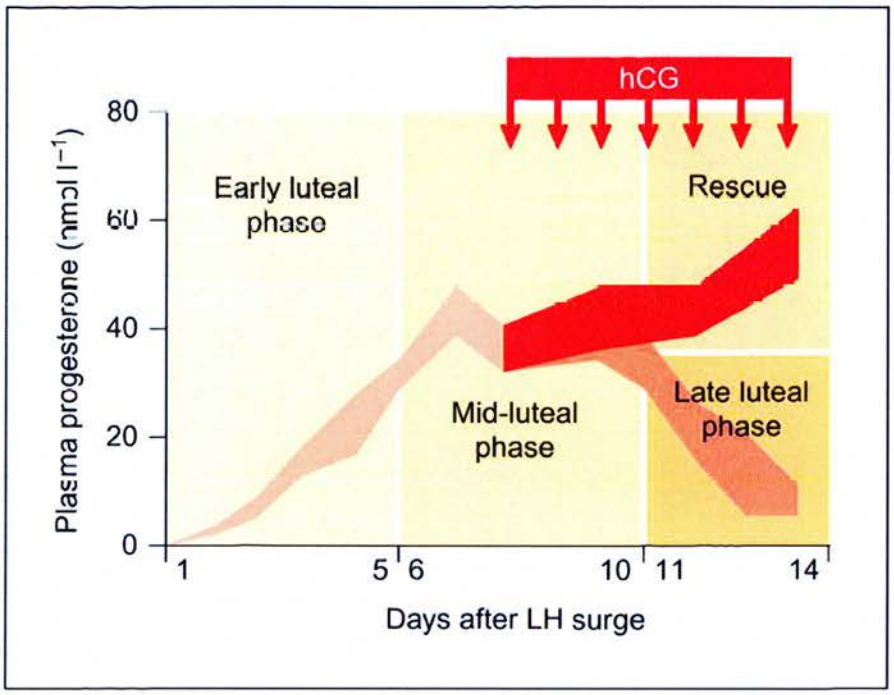


Figure 2.1 Schematic representation of plasma progesterone concentrations in carefully dated human corpora lutea. Progesterone levels rapidly rise in the early-luteal phase (LH+1 to LH+5), peak in the mid-luteal phase (LH+6 to LH+10) and fall in the absence of pregnancy hormone during the late-luteal stage (LH+11 to LH+14). However, after luteal 'rescue' progesterone concentrations are maintained and increased from LH+11 to LH+14.

(Duncan, 2000)

2.1.2.6 Derivation of primary cell culture from patients undergoing assisted conception programmes

Primary cell culture material was obtained from women undergoing assisted conception programmes in EFREC at the Royal Infirmary of Edinburgh. Follicular fluid was collected from women undergoing transvaginal oocyte retrieval for *in vitro* fertilisation procedures. Ovarian stimulation was achieved by a long-protocol stimulated cycle which involved intranasal naferelin (Pharmacia Biotech, Milton Keynes, UK) to down-regulate pituitary gonadotrophins, followed by daily purified gonadotrophins (Menopur, Ferring

Pharmaceutical, Langley, UK) for ovarian stimulation. When at least three follicles reached 18 mm in diameter, 10,000 IU hCG was administered and 35 h later transvaginal oocyte collection was performed under sonographic guidance. After the removal of the oocyte, follicular fluid was aspirated from the remaining follicles. Individual follicles were not distinguished and all follicular fluid from the same patient was pooled. Samples were stored at 4°C after operations until transfer to the laboratory on the same day.

2.1.3 Luteinised granulosa cells

Collected follicular fluid was carefully transferred into 50 ml centrifuge tubes and spun at 1500 rpm for 10 min. After supernatant was removed, the cellular layer was layered over a 45% Percoll/serum-free culture medium mixture and centrifuged at 1200 rpm for 30 min in order to pellet blood cells. Luteinised granulosa cells were then visible in the interface and were carefully pipetted from Percoll solution and placed in a clean 20 ml centrifuge tube for subsequent washes with sterile phosphate buffered saline (PBS) (Invitrogen, Paisley UK). Once the cells had 1x 10 min wash and at least 3x 3 min washes, they were resuspended in 1-2 ml (depending on size of pellet) of serum-free culture medium and viable cells were counted on a haemocytometer using a trypan blue (Sigma-Aldrich, Dorset UK) exclusion method. Twenty-four well cell culture plates were precoated with 25 µl of matrigel (BD Biosciences, Bedford, MA) diluted 1:1 with serum-free culture medium before cells were plated. Once the Matrigel had set, each well was briefly washed with culture medium before plating approximately 100,000 viable cells per well in 1 ml of serum-free media. Cell were incubated at 37°C in 5% CO_2 in air for 5-7 days or as otherwise described.

2.1.3.1 Manipulation of hCG in prolonged cultures of luteinised granulosa cells

In order to mimic the luteal phase in primary cell culture, luteinised granulosa cells were plated as described above (section 2.1.3) and grown for 12 days. Cells were stimulated with low dose hCG (1 ng/ml) with LDL (50 mg/l) beginning on day 2 and this was repeated every 2nd day until day 7 when treatments were replaced with maximal doses of 100 ng/ml hCG/LDL or LDL alone. Cells were analysed after seven days with hCG and on day 12 in the presence or absence of hCG to mimic the progesterone secretion profile of late-luteal and luteal rescue stages respectively (Duncan *et al.*, 2005a).

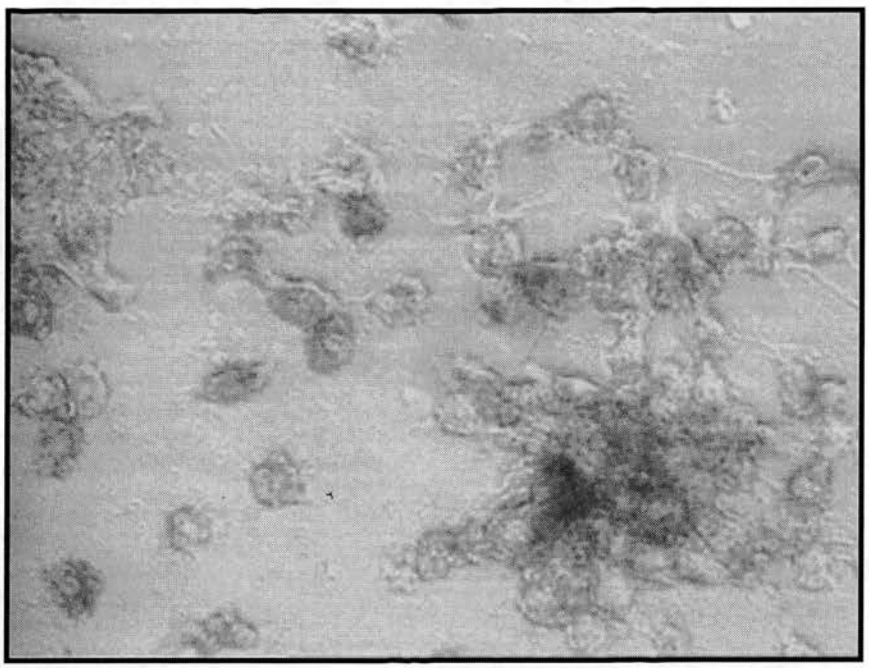


Figure 2.2 Phase-contrast image of luteinised granulosa cells in primary cell culture.

2.1.4 Fibroblast-like cells

To derive ovarian fibroblast-like cells, collected follicular fluid was carefully transferred into 50 ml centrifuge tubes and centrifuged at 1500 rpm for 10 min as above. Supernatant fluid was then removed and the cellular component was resuspended in 5 ml of 10% fetal bovine serum (FBS) supplemented culture media before being carefully pipetted into a plastic cell culture flask. Medium was changed weekly and cells were trypsinised (Sigma) after 2 weeks in order to remove contaminating granulosa cells that were at the end-stage of their life. Once fibroblast-like cells had reached confluence (Figure 2.3), usually around 5-7 weeks, they were removed from the flasks using trypsin/ethylendiaminetetraacetic acid (EDTA) solution and viable cells were counted on a haemocytometer using a trypan blue exclusion method. Sixty-thousand cells per well were then plated in 1 ml of FBS culture medium on 24-well plastic cell culture plates and used for investigation as described in appropriate chapters.

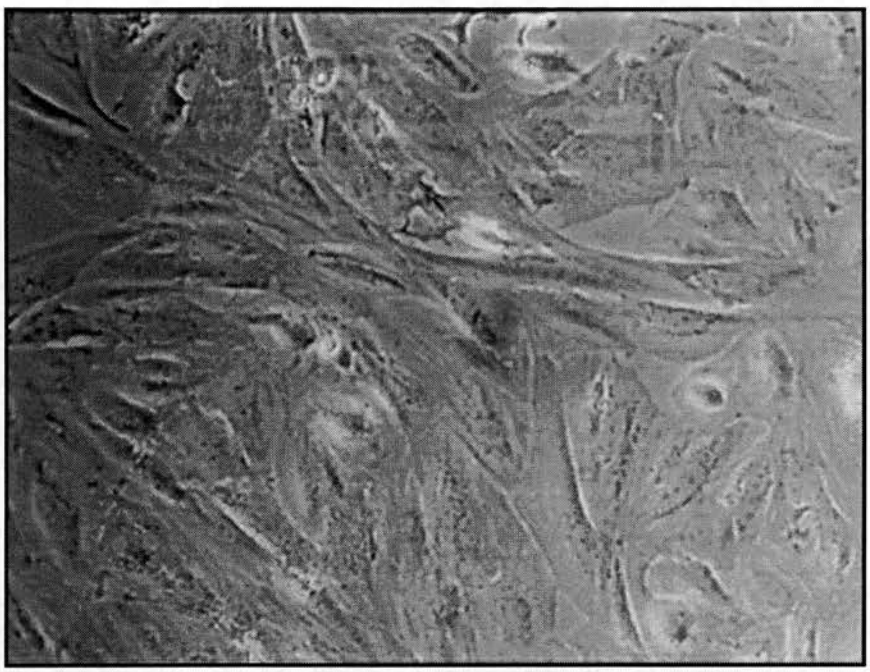


Figure 2.3 Phase contrast image of fibroblast-like cells in primary cell culture.

2.1.5 Tissue preservation

Upon collection of human corpora lutea, two quarters of the tissue were immediately frozen and stored at -70 C until RNA extraction was carried out. The remaining tissue was fixed in 10% neutral buffered formalin.

2.1.6 Tissue fixation

In order to preserve tissue and cell preparations in a life-like manner, fixatives were used stabilize the morphology and protect them against the rigors of processing and staining techniques. The fixative used for preparations described in this study was 10% neutral buffered formalin.

2.1.6.1 Human corpora lutea

Human corpora lutea sections were fixed 10% neutral buffered formalin at the time of collection and left in solution for 24 h. Tissue was then transferred to 70% ethanol and

processed for 17.5 h in an automated Leica TP1050 processor (Leica Microsystems, Milton Keynes, UK). Processed tissue was embedded by hand in liquid paraffin wax and the cooled wax block was stored at room temperature until required.

2.1.7 Histological analysis of tissue

Gross histological analysis of tissue or cell morphology was performed on hematoxylin and eosin stained sections. Sections were dewaxed as detailed in section 2.1.9.2 and immersed in Harris's haematoxylin for 3-5 min which stains nuclei purple. Slides were then briefly washed in water before being de-differentiated in 1% acid alcohol very briefly and washed again in water. To allow cell nuclei to 'blue up' sections were placed in Scott's tap water for up to 1 min before being immersed in Eosin for 30 s for cytoplasmic staining. Slides were washed in tap water before dehydration and cover slipping took place as described in section 2.1.9.8.

2.1.8 Protein analysis

2.1.9 Immunohistochemistry

In order for a stain or a particular protein to more readily seen with a microscope, an antibody can be used to link a cellular antigen specifically to the protein. This technique is known as immunohistochemistry in tissues and immunocytochemistry in cells. With the abundance of a variety of offered antibodies and several detection methods available, optimal conditions for immunohistochemical detection must be determined for individual situations. Immunohistochemistry was performed as detailed below on sections of human corpora lutea using standard detection protocols to highlight localisation of protein of interest.

2.1.9.1 Sectioning

Fixed tissue was routinely processed to paraffin and subsequently embedded in wax blocks. Prior to sectioning, blocks were chilled on ice to make them more rigid and easier to cut into 5 μm sections using a Leica microtome (model RM 2135). Cut sections were floated on a waterbath (Lamb RA, model E/65) pre-set at 45°C in order to flatten

sections and remove any folds or creases before mounting on poly-L-lysine coated slides (VWR, Lutterworth, Leics, UK). Sections were then placed in slide racks and dried overnight in an oven set between 40-60°C.

2.1.9.2 Dewaxing and rehydrating

Sections were deparaffinised in Histoclear (National diagnostics, Hull, UK) at room temperature for 5 min, x2. Tissue was then rehydrated by immersing in alcohols beginning at 100% ethanol for 30 s x2, 95% for 30 s, 75% for 30 s before being washed in tap water.

2.1.9.3 Antigen retrieval

In order to maximise the availability of the tissue antigen to interact with specific antibodies an antigen retrieval method was employed. In some cases fixatives used to preserve tissue integrity can mask proteins by over inducing cross-linkings of the tissue and thus they are no longer recognised by their complimentary antibodies. To overcome this problem, sections were immersed in a high temperature antigen retrieval method in 2L of citrate buffer, pH 6.0 in a domestic pressure cooker (Tefal, Clypso). Once boiling, sections were placed into cooker for 5 min of full pressure and left to stand for approximately 20 min before being cooled under running water.

2.1.9.4 Blocking

Non-specific binding is a common problem encountered during immunohistochemical localisation. This is usually due to a primary or secondary antibody or other detection reagents binding non-specifically to molecules other than the desired protein.

2.1.9.4.1 Endogenous peroxidase blocking

Horseradish peroxidase (HRP) amplication was used (see section 2.1.9.6) to catalyse a colourmetric result for antibody detection and consequently endogenous peroxidase activity needed to be blocked. Sections were immersed in 3% hydrogen peroxidase in methanol (30% H_2O_2, VWR) for 30 min at room temperature on a rocker after antigen retrieval. Sections were then washed in water for 5 min.

2.1.9.4.2 Endogenous biotin blocking

As biotin is a co-factor for a number of enzymes within the body and has wide tissue distribution, blocking of endogenous biotin can be an important step when using biotinylated secondary reagents (see section 2.1.9.6). Endogenous biotin can be sequestered by the use of an avidin/biotin kit (Vector Laboratories, Orton Southgate, UK). Avidin is incubated on sections for 15 min to bind any endogenous biotin and a subsequent application of biotin for 15 min is added to fill any of the binding sites on the avidin molecule.

2.1.9.4.3 Non-specific binding of secondary antibody blocking

In order prevent any non-specific binding of secondary antibodies, sections were incubated in a dilute solution from the species' in which the secondary antibody was raised in. In the majority of cases a goat or rabbit raised secondary antibody was used and therefore goat or rabbit serum respectively was used. Animal serum (Diagnostics Scotland, Edinburgh UK) was diluted 1 in 5 in PBS containing 5% bovine serum albumin (BSA; Sigma). Sections were removed from water after peroxidase blocking and carefully dried around tissue section to remove excess liquid. Blocking serum was added to sections which were incubated in a humidity chamber at room temperature for approximately 1 h.

2.1.9.5 Primary antibodies

After appropriate serum blocking, slides were gently tapped and carefully wiped around tissue section to remove serum which was then replaced with a solution of primary antibody. Primary antibodies were diluted in blocking serum at an optimised concentration. Slides were incubated overnight in a humidity chamber at 4°C. Negative control sections were incubated with blocking serum alone as the primary antibody was omitted to confirm antibody specificity.

2.1.9.6 Secondary antibodies

Once the primary antibody was washed off, the appropriate secondary antibody was incubated on section in order to amplify the detection of the primary antibody. Secondary antibodies raised against a species-specific sequence on the primary antibody, labelled with biotin were diluted 1 in 500 in blocking serum and incubated on sections at room

temperature for 1 h in a humidity chamber. To amplify antibody signal, sections were then washed and incubated for 1 h with avidin-biotin HRP complex (ABC-HRP; DAKO) which takes advantage of the extremely high binding affinity that avidin and biotin have for each another. ABC-HRP complex was made according to manufacturers' instructions at least 30 min prior to use.

2.1.9.7 Antigen detection

To localise the desired antigen, liquid 3,3'-diaminobenzidine (DAB; DAKO, Cambridgeshire UK) was used to generate a colourmetric result. DAB chromagen was diluted in the provided buffer according to manufacturers' instructions just prior to use. Once DAB staining was optimised in a positive control section, subsequent sections were incubated in the reagent for the desired time. Generally this time varied from 30 s to 3 min depending on the primary antibody used. Sections were placed in distilled water in order to stop the reaction and a colour was generated at the site of the original antigen, therefore allowing its localisation within the tissue.

2.1.9.8 Hematoxylin & eosin counterstain and mount

Sections were lightly counterstained in Harris's hematoxylin in order to stain nuclear tissue. Nuclei initially stain a purple colour until washed in water and immersed in Scott's tap water for 1 min after which they will appear blue. In order to redehydrate the tissue, sections were immersed in 70% ethanol for 20 s, 85% ethanol for 20 s, 95% ethanol for 20 s and 100% ethanol for 20 s x2. To prepare sections for mounting they were then immersed in HistoClear (National diagnostics) for 5 min and cleared in Xylene for 5 min. Glass coverslips (VWR, Lutterworth, UK) were mounted on sections using the solvent based glue, Pertex (Cell Path, Hemel Hempstead, UK). Slides were left to dry in a fume hood until used for light microscopy.

2.1.9.9 Light microscopy

Images were captured using an Olympus Corp. Provis microscope (Olympus Corp. Optical Co., London, UK) equipped with a Kodak DCS330 camera (Eastman Kodak Co., Rochester, NY), stored as a digital file and assembled using Photoshop CS2 (Adobe Systems Inc. Mountain View, CA).

2.1.10 Immunofluorescence

Another detection method, and one that allows co-localisation of more than one antigen, is fluorescent immunohistochemistry. Sections were dewaxed, rehydrated and if necessary antigen retrieved as previously described (section 2.1.9.3). Sections only required peroxidase blocking if Tyramide Signal Application (TSA; Perkin-Elmer Life Sciences, Boston, USA) enhancement was employed as this method involves the use of a secondary antibody which is conjugated to HRP complex (section 2.1.10.2.3).

2.1.10.1 Primary antibody

After appropriate serum blocking, slides were gently tapped and carefully wiped around tissue section to remove serum which was then replaced with a solution of primary antibody. Primary antibodies were diluted in blocking serum at an optimised concentration. Slides were incubated overnight in a humidity chamber at 4°C. Negative control sections were incubated with blocking serum alone as the primary antibody was omitted to confirm antibody specificity.

2.1.10.2 Secondary antibody

Sections were washed in PBS after primary antibody incubations and the appropriate secondary antibody was added for 1 hr. Fluorescent chromagens are light sensitive and, once added, slides were protected from the light to prevent bleaching of the signal.

2.1.10.2.1 Direct method

Dectection with an Alexa fluorescent dye was a single-step process whereby sections could be incubated with the secondary antibody that is directly conjugated to a fluorescent chromagen.

2.1.10.2.2 Indirect method

This method was a two-step process that allowed amplification of immunofluorescence via a biotin labelled secondary antibody. Sections were incubated with a biotin labelled secondary antibody that was raised against a species-specific sequence on the arm of the primary antibody. The signal was amplified and detected using a fluorescently labelled avidin or strepavidin complex which binds to the biotin of the previous antibody.

2.1.10.2.3 Tyramide enhancement method

The tyramide Cy3 (TSA plus cyanine 3 system; Perkin-Elmer Life Sciences) was used in order to amplify a primary antibody signal diluted 1 in 50 in the supplied buffer. This detection method significantly increases the sensitivity of the primary antibody without increasing the resolution or background staining.

2.1.10.3 Counterstain and mount

For labelling of nucleic acids sections were incubated in either To-Pro 3 (Molecular Probes, Invitrogen) as indicated in appropriate chapter. To-Pro results in nuclear-specific stains of blue. After nuclear stain sections were washed in PBS and mounted in Permafluor aqueous mounting media (Beckman Coulter, High Wycombe, UK).

2.1.10.4 Fluorescent Microscopy

Fluorescent images were captured using an LSM 510 Axiovert 100M confocal microscope (Carl Zeiss Ltd., Welwyn Garden City, UK). Images were compiled using Photoshop CS2 (Adobe Systems Inc.).

2.1.11 Gelatine Zymography

2.1.11.1 Freeze drying of collected cell culture medium

Cell culture medium was collected from serum-free cultures and subsequently frozen at -20°C. Aliquots of 200 µl were subjected to freeze drying for 2-3 h until they resembled a powder and then reconstituted in 20 µl of sterile dH_2O.

2.1.11.2 Preparation of polyacrylamide gels containing gelatine

Gelatine zymography was prepared and run with a BioRad mini PROTEAN 3 (BioRad, Hercules, CA, USA). Sodium dodecyl sulfate (SDS) gels were prepared in two parts; a resolving lower gel that contained 10 mg/ml gelatine (w/vol) and 0.1% SDS and a 0.1% SDS upper stacking gel that contained the loading wells. Once the resolving gel was poured between the glass apparatus, a thin layer of water saturated sec-butanol (1:1vol/vol) was carefully overlayed to prevent bubbles and level up the top of the gel. The resolving gel was then left to set at room temperature for 45 min before the stacking gel was poured on and left to set at room temperature for 90 min.

2.1.11.3 Gel Electrophoresis

One microlitre of reconstituted sample was added to sample buffer and gels were run in 1x SDS buffer at 100 V for 1.5 h. A molecular weight marker (Perfect Protein Marker, Novagen, WI, USA) was used to identify protein size and an internal control was used in every zymography performed to ensure gel compatibility. Gels were then incubated in 2.5% Triton X-100 for 45 min after electrophoretic protein separation washed in tris-buffered saline (TBS) wash buffer and incubated overnight at 37°C in digestion buffer to facilitate enzymatic activity.

2.1.11.4 Identification of gelatinase activity

Once digestion incubation was complete, gels were briefly washed and immersed in a acetic acid/methanol solution containing 0.5% (wt/vol) Coomassie brilliant blue for 1 h. Gels were then detained using the same solution minus the Coomassie dye for 30 min or as long as it took for the white bands to appear which reflect the MMP-2 activity. Gels were stored in water until analysed by transmission densitometry (G-700 densitometre; BioRad, Hertsfordshire, UK) using an integrated software program (Quantity One, BioRad).

2.1.12 RNA analysis

2.1.13 RNA extraction

RNA was extracted using two different methods throughout this study. Tri-Reagent (Sigma) RNA extraction methods were employed for archival corpora lutea that had been extracted before the current study. Additionally, RNA extracted from primary cell culture in Chapter 3 also extracted using Tri-Reagent. Alternatively, RNA extractions performed in all remaining chapters and from archival frozen corpora lutea was extracted using RNA Mini extraction kits (Qiagen, Crawley, UK).

2.1.13.1 RNA extraction using Tri-Reagent

Total RNA was extracted from 0.2-0.5g frozen corpora lutea samples or cultured primary cell using Tri-Reagent according to manufacturers' instructions. For every 1 ml of Tri-

Reagent, 200 µl of chloroform was added to sample, shaken vigorously and let to stand at room temperature for 5 min. Samples were centrifuged at 13,000 rpm at 4°C for 20 min so that the top layer containing the RNA portion could be carefully removed and placed in a clean tube containing 500 µl isopropanol. Samples were gently mixed and left on ice at 4°C for 1 h and centrifuged at 13,000 rpm at 4°C for 15 min. With a pellet now visible, isopropanol was carefully removed and replaced with 500 µl of 70% ethanol, vortexed and centrifuged at 13,000 for 10 min at 4°C. In order to resuspend pellet in nuclease free water, ethanol was removed and pellet was left for 5 min to dry. Generally cell preparations were resuspended in 10 µl of nuclease free water whereas tissue material was resuspended in greater volumes.

2.1.13.2 RNA extraction using RNeasy Mini extraction kits

Non-extracted archival and more recently collected human corpora lutea, along with cultured primary cell RNA was extracted using Qiagen RNeasy Mini kits (Qiagen). Frozen archival corpora lutea was thawed in enough RNA later solution (Qiagen) to cover tissue in order to prevent degradation before being immersed in lysis buffer supplemented with 10 µl/ml β-mercaptoethanol. Tissue was then homogenised (Qiagen Tissue Lyser) for 2x 3 min at 30 Hz before proteinase K (Qiagen) digestion step at 55°C for 10 min. Cultured cell isolations were not subjected to these steps and once cells were harvested in lysis buffer/ β-mercaptoethanol solution they were vortexed and stored at -70°C until extraction took place.

From this step onwards extraction protocol for tissue and cells was the same. Suspensions were diluted with an equal volume of 70% ethanol and applied to the mini columns supplied in kit. Columns were washed with supplied buffers and treated with DNase solution to remove any contaminating genomic DNA. DNase was then inactivated by and removed with washing buffers before elution buffers were used. RNA was eluted twice from columns with 30 µl of RNase-free water.

2.1.14 RNA quantification

2.1.14.1 RNA quantification using the NanoDrop ND-1000

RNA was quantified using the NanoDrop ND-1000 Spectrophometer (NanoDrop Technologies, Wilmington, USA) which calculates the RNA concentration according to

the absorbance measured at 260 nm. The integrity of the RNA could also be determined using the 260/280 nm ratio with a ratio of 1.8-2 indicating the best quality.

2.1.14.2 RNA quantification using the Agilent 2100 Bioanalyser

To double check the integrity of RNA (especially important regarding archival tissue) the Agilent 2100 Bioanalyser (Agilent Technologies,) was used in conjunction with a RNA 6000 Nano chip. Unlike the NanoDrop method this method displays the ratio of intact ribosomal RNA peaks (18S/28S) to measure the RNA concentration and integrity.

2.1.15 DNase treatment

RNA that had not been extracted using the Qiagen Mini kits was subjected to DNase treatment using a Promega (Southampton, UK) protocol mix displayed in Table 2.1. RNA was incubated in a digestion solution to inactivate any contaminating DNA at 37°C for 30 min before a stop solution was added to samples to terminate the reaction. Samples were then incubated at 70°C for 10 min to inactivate the DNase before being reverse transcribed. Negative controls were included with nuclease-free water substituted for either the RNA or DNase enzyme.

Table 2.1 Reagents used for DNase treatment protocol

Mix reagents	**1x**
RNA	1-8 µl
DNase 10x Buffer	1 µl
RNase Inhibitor	0.1 µl
DNase enzyme (1 unit/µl)	1u/µg RNA
Nuclease-free water to a final vol of	10 µl

2.1.16 Reverse transcription

Reverse transcription (RT) was performed under carefully controlled conditions using a Taqman Reverse Transcription kit with random hexamers (Applied Biosystems,

Warrington, UK). A mastermix of the reagents was prepared for the desired number of reactions and are shown below in Table 2.2.

Table 2.2 Reagents used for reverse transcription reaction

Mix reagents	**1x**
Nuclease-free water	1.85 µl
10x Taqman buffer	1.0 µl
$MgCl_2$ (25 mM)	2.2 µl
dNTPs (10 mM)	2.0 µl
Random Hexamers	0.5 µl
RNase Inhibitor	0.2 µl
Multiscribe reverse transcriptase	0.25 µl x n-0.25

Before the multiscribe enzyme was added to the reaction mixture, 7.75 µl of the mastermix was removed and used as a negative RT control. Therefore the volume of enzyme required was 0.25 µl x the number of reactions (n) -0.25 µl. The mastermix was vortexed and 8 µl aliquots for single reactions or 16 µl aliquots for double reactions were placed in 0.2 ml Eppendorf tubes followed by 2 µl or 4 µl of RNA respectively. A RT H_2O water negative was also included and the appropriate volume of nuclease-free water was added in place of RNA. The reverse transcription reaction took place in a Thermocycle Eppendorf polymerase chain reaction (PCR) machine at the following temperature cycles; 25°C for 1 h (incubation), 48°C for 45 min (reverse transcription step) and 95°C for 15 min (denaturing step).

2.1.17 Primer design and validation for semi-quantitative and quantitative RT-PCR

Oligonucleotide primers for genes investigated using conventional PCR or Lightcyling quantitative RT-PCR (qRT-PCR) were designed using the Primer 3 software (http://primer3.sourceforge.net/) from DNA sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez). Primers were synthesised by either MWG-AG Biotech (Milton Keynes, UK) or Eurogentec (Southampton, UK). All 5' to 3' sequences are listed in appropriate chapters. Primer probe sets used for qRT-PCR Taqman analysis were either designed using Primer Express (Applied Biosystems)

program or obtained from previous publications, sequences verified using GenBank and validated to ensure that the amplification efficiencies of target gene and internal standard are approximately equal.

2.1.18 Conventional PCR

2.1.18.1 cDNA amplification

Amplifaction of cDNA was carried out using Taq DNA polymerase (Promega) using the mastermix reaction listed in Table 2.3.

Table 2.3 Reaction mixture for cDNA amplification

Mix reagents	**1x**
Nuclease-free water	4.7 µl
10x buffer	1.0 µl
MgCl2 (25 mM)	0.6 µl
dNTPs (10 mM)	0.2 µl
Forward primer (5 µM)	1.0 µl
Reverse primer (5 µM)	1.0 µl
Taq polymerase (5 u/µl)	0.5 µl

Once all the mix reagents were combined, vortexed and aliquoted into 0.2 ml Eppendorf tubes, 1 µl of appropriate cDNA was added to each tube to make a final volume of 10 µl for each reaction. Negative controls were exactly the same as sample tubes with nuclease-free water in place of cDNA sample. PCR conditions using a Thermocycle Eppendorf PCR machine were as follows; incubation 95°C for 5 min, 35 cycles of 95°C for 30 sec, appropriate annealling temperature (primer dependent) for 30 sec, and 72°C for 90 sec, followed by 72°C for 10 min. Appropriate theoretical annealing temperatures (Tm) were calculated for each set of primers using the formula;

Tm= 2°C x (number of A and T residues) + 4°C x (number of G and C residues) -5°C

2.1.18.2 Gel electrophoresis

PCR products were separated by size fractions using gel electrophoresis in the presence of ethidium bromide. PCR products were mixed with a 5x loading dye in a 1:5 ratio before being loaded into a 2% agarose gel (2.4 g agarose, 120 ml 1x tris-acetae-EDTA (TAE) buffer, 2 µl ethidium bromide) in 1x TAE. Product sizes were verified using a

100-base pair ladder which acts as a reference size marker. Electrophoresis was run at 110 V for 1 h and visualised and photographed under UV transillumination.

2.1.19 Quantitative PCR

Two methods were used in the current study to quantify gene expression of human corpora lutea and cultured cells.

2.1.19.1 Light cycling qRT-PCR

Each light cycling assay was optimised using placental cDNA to determine annealling temperature and $MgCl_2$ concentrations favourable for each gene of interest. Eight PCR reactions were performed as explained above in section 2.1.18.1 using the gradient facility of the PCR cycler to demonstrate the effectiveness at annealing temperatures. PCR products were examined by gel electrophoresis as detailed in section 2.1.18.2 to confirm the presence of a single band at the correct size (products for light cycling usually range from 150-200 bp). Optimal $MgCl_2$ concentrations were determined on the Roche LightCycler machine (Roche, Lewes, UK) with test range from 3-5 mM concentrations. A melting curve analysis was also used to determine the optimal temperature that would be used to quantify the level of gene expression by minimising background fluorescence from non-specifically amplified DNA. Therefore all assays had to demonstrate a single specific melting curve peak in order to be considered for subsequent analysis.

A standard curve was generated with serial dilutions of standardised cDNA using the second derivative maximum method provided with the LightCycler software (version 3.3). Reaction mixtures were set up using the Mastermix supplied with the LightCycler Fast StartNDAMaster SYBR Green 1 kit (Roche) utilising the predetermined optimal conditions. Reaction mixures are shown in Table 2.4. All reactions were performed in 10 µl volume duplicates. All runs had negative controls whereby nuclease-free water was substituted for cDNA.

Table 2.4 Reaction mixture for LightCycler qRT-PCR

Mix reagents	**1x**
SybrGreen mastermix	1 µl
Forward primer	1 µl
Reverse primer	1 µl
$MgCl_2$ (3-5 mM)	2 µl (up to)
cDNA	1 µl
Nuclease-free water	4 µl (up to)

Concentrations for each gene of interest were automatically calculated using the computer software which compares the sample threshold crossing point reference to the simultaneously generated standard curve. Ideally, the level of each gene expression should lay within the boundaries of the corresponding standard curve. Variation for each assay was typically within 10% and the same placental cDNA sample was used for corresponding assays. All data was normalised according to the gene expression levels of glucose-6-phosphate dehydrogenase (G6PDH) determined in duplicate by reference to a serial dilution calibration curve that was generated for each sample using the LightCycler software.

2.1.19.2 Taqman ® qRT-PCR

Unlike the Lightcycler qRT-PCR method, Taqman involves an oligonucleotide probe in addition to the forward and reverse primer sequences. The Taqman probe which has a fluorescent reporter dye attached to the 5'-end and a quencher moiety coupled to the 3'-end is carefully designed to hybridise to an internal region of the PCR product. When the probe is intact, no fluorescence is omitted as the reporter dye emission is quenched. However, during the PCR reaction in the presence of DNA polymerase the probe is cleaved, and once separated from the quencher, the reporter dye emits fluorescence. Therefore, the emission of fluorescence will increase in proportion to the number of cycles.

Table 2.5 Reaction mixture for Taqman qRT-PCR

Mix reagents	**1x**
Taqman Universal mastermix	12.5 µl
18S primer/probe	0.375 µl
Forward primer	0.3 µl
Reverse primer	0.3 µl
Nuclease-free water	8.525 µl
Gene probe	1.0 µl

The mix listed above in Table 2.5 was vortexed well, aliquoted into 46 µl volumes per tube before 4 µl of cDNA was added to each. Tubes were mixed again and 23 µl portions were added in duplicates to 96-well optical reaction plates. A PCR water control and an RT-negative control were set up by replacing the cDNA with 4 µl of nuclease-free water and 4 µl of RT-negative sample respectively. Plates were sealed with optical film and run out on the ABIPRISM® 7900 heat-cycler system. PCR cycles were in four stages; 50°C for 2 min, 95°C for 10 min, 95°C for 15 min (40 cycles) and 59°C for 1 min.

Data was analysed using the ABIPRISM® 7900 software and calculate using the comparative Ct ($2^{-\Delta\Delta Ct}$) method which calculates the changes in gene expression as a relative fold difference between experimental and calibrator sample.

2.1.20 Commonly used solutions

2.1.21 Cell culture solutions

ITS Serum-free Culture Medium

Insulin (6.25 mg/l), transferrin (6.25 mg/l), selenious acid (6.25 µg/l) (ITS)	5 ml
Amphoteracin (2.5 mg/l)	5 ml
Penicillin (50 mg/ml)	5 ml
Streptomyocin (60 mg/l)	5 ml
Dulbecco's minimum essential medium/F12 Ham mixture	480 ml

10% Fetal Bovine Serum (FBS) Culture Medium

10% FBS	50ml
Amphoteracin (2.5 mg/l)	5 ml
Penicillin (50 mg/ml)	5 ml
Streptomyocin (60 mg/l)	5 ml
Dulbecco's minimum essential medium/F12 Ham mixture	435 ml

0.1% BSA in PBS

0.1% w/v BSA	5 mg
PBS	5 ml

Matrigel Basement Membrane Matrix

50% v/v matrigel	400 µl
ITS culture medium	400 µl

2.1.22 Immunohistochemistry Buffers

PBS

PBS tablets (Sigma)	5 tablets
Water	1 L

PBS/Tween

PBS	1L
Tween20	100 µl
NaCl	8 g

TBS 0.5M

Tris	60.5 g
NaCl	87.6 g
HCl	300 ml
Water	up to 10 L

2.1.23 Gelatin zymography

2.1.23.1 Acrylamide gels

Resolving gel (recipe for 2 gels)

Water	3.85 ml
Gelatin (10 mg/ml water)	1.0 ml
Resolving gel buffer	2.5 ml
10% SDS	100 µl
10% Ammonium Persulfate	50 µl
30% Acrylamide/Bis	2.5 ml
TEMED	5 µl

Stacking gel (recipe for 2 gels)

Water	3.05 ml
Stacking gel buffer	1.25 ml
10% SDS	50 µl
10% Ammonium Persulfate	50 µl
30% Acrylamide/Bis	0.65 ml
TEMED	5 µl

2.1.23.2 Buffers

Sample Application Buffer

20% v/v Glycerol	10 ml
2% w/v SDS	1 g
0.04% w/v Bromophenol Blue	20 mg
Water	50 ml

Running Tank Buffer x10 (pH 8.3)

0.25 M Tris	30.3 g
1.9 M Glycine	144 g
1% SDS	10 g
Water	up to 1 L

Resolving Gel Buffer (pH 8.8)

1.5 M Tris	18.3 g
Water	100 ml

Stacking Gel Buffer (pH 6.8)

0.5 M Tris	6.06 g
Water	100 ml

Stock Wash Buffer x10 (pH 8.0)

0.5 M Tris	60.57 g
1.5 M NaCl	90 g
Water	1 L

Triton X-100 Wash

2.5% Triton X-100	5 ml
X1 TBS	200 ml

Digestion Buffer (pH 7.6)

50 mM Tris	6.07 g
0.2 M NaCl	11.69 g
5 mM CaCl2	735 mg
1 μM ZnCl2	1 ml of 1 mM sol
0.02% Brij-35 (30% sol)	660 μl
Water	up to 1 L

Destaining Solution

30% Methanol	300 ml
10% Glacial Acetic Acid	100 ml
Water	600 ml

Staining Solution

0.5% w/v Coomassie Brilliant Blue	1 g
Destaining Solution	200 ml

Sodium Dodecyl Sulphate (SDS)

10% w/v SDS	10 g
Water	100 ml

Gelatin

10 mg/ml w/v Gelatin	0.25 g
Water	25ml

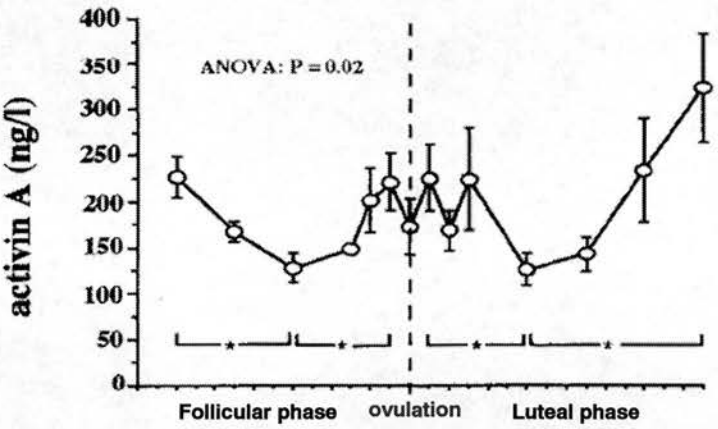
Ammonium Persulfate

10% w/v Ammonium Persulfate	50 mg
Water	500 µl

sec-Butanol

Butanol	50 ml
Water	50 ml

3 *In vitro* evidence suggests activin A may promote tissue remodelling associated with human luteolysis



Activin A is a dimeric glycoprotein that is maximal during the late-luteal phase in the peripheral serum of naturally cycling women. Interestingly, these high concentrations of activin A correlate with the time when MMP-2 is also at the peak of its activity. MMP-2 is an important molecule associated with luteolysis not only in women, but in many other species. Therefore, the focus of this chapter is to determine if activin A is paracrine regulator of the tissue remodelling associated with luteolysis.

(Peripheral serum concentrations in naturally cycling women from Muttukrishna *et al.*, 1996)

3.1 Introduction

The human corpus luteum is a highly vascular and active endocrine gland that in the mid-luteal phase measures up to 2 cm in diameter. However, unless hCG is secreted by a conceptus, this highly transient structure will undergo functional and structural luteolysis, (Behrman *et al.*, 1993) becoming a small fibrous remnant within a matter of days. The process of luteolysis is associated with marked tissue remodelling and vascular involution. Cellular processes that are known to occur during the regression of the human corpus luteum include cell death (Fraser *et al.*, 1999), an increase in the expression of connective tissue growth factor (CTGF) (Duncan *et al.*, 2005b), an influx of macrophages (Duncan *et al.*, 1998b) and an up-regulation of fibroblast MMP-2 expression and activity (Duncan *et al.*, 1998a).

MMPs are key proteolytic enzymes involved in the degradation of the ECM, which is constantly remodeled during luteolysis. Much evidence exists to suggest that MMP-2 is an important luteolytic agent during the demise of the corpus luteum. In rats, structural luteolysis was associated with an increase in MMP-2 activity (Endo *et al.*, 1993) whilst in pigs the expression of MMP-2 was elevated in the regressing corpus luteum (Pitzel *et al.*, 2000). Additionally, primates (Young *et al.*, 2002) and humans (Duncan *et al.*, 1998a) show maximal MMP-2 expression in the late-luteal phase, positively correlating with the functional and structural regression of the corpus luteum. Furthermore hCG during maternal recognition of pregnancy in women (Duncan *et al.*, 1998a) reduces the expression of MMP-2. As MMP-2 is up-regulated during luteolysis, and conversely inhibited in the presence of hCG during maternal recognition of pregnancy, it is likely that it has an important role in the luteolytic process.

The primary source of MMP-2 in the human corpus luteum is the luteal fibroblasts that do not express LH/hCG receptors (Duncan *et al.*, 1998a), suggesting that there is a paracrine regulator of MMP-2 expression (Duncan, 2000). Therefore the present study hypothesised that in the corpus luteum, MMP-2 is regulated by hCG through an intermediate molecule. Unlike LH, FSH, progesterone, oestradiol or inhibin A, the concentrations of activin A in serum are maximal during the late-luteal phase (Muttukrishna *et al.*, 1996), suggesting it may have a positive luteolytic action. Activins and other members of the TGF-β superfamily are known to control many diverse

physiological processes (Massague, 1998), therefore in this study it was hypothesised that activin A may be a critical intermediate signalling molecule in the human corpus luteum.

As described previously (Duncan *et al.*, 1996a; Duncan *et al.*, 1998a; Duncan *et al.*, 1998b), this laboratory has developed a system for the collection of carefully dated human corpora lutea and more recently (Duncan *et al.*, 2005b) a primary cell culture system using human luteinised granulosa cells and/or novel cultures of fibroblasts-like cells derived from the luteinising follicle. The aim of the current study was to investigate whether activin A was a paracrine regulator of luteal tissue remodelling by investigating its effects on MMP-2 expression and activity using *in vitro* model systems.

3.1.1 Aims

To establish the source of activin A in the human corpus luteum and primary cultures of luteinised granulosa cells

To establish an activin signalling pathway and subsequent reception in the human corpus luteum and primary cultures of luteinised granulosa cells

To understand the changes in activin signalling that occur in the human corpus luteum

To establish and characterise an appropriate *in vitro* model to study the paracrine interactions that occurs between neighbouring cells in the human corpus luteum

To understand the effects of activin A on the human corpus luteum using the *in vitro* system to investigate a potential paracrine signalling role

3.2 Materials and Methods

3.2.1 Collection of human corpora lutea

Human corpora lutea (n=18) were collected from women with regular menstrual cycles undergoing hysterectomy for benign conditions and dated on the basis of the urinary LH surge as fully described in section 2.1.2.2. In this study, six corpora lutea were classified as early-luteal (LH+1 to LH+5), six as mid-luteal (LH+6 to LH+10), and six as late-luteal (LH+11 to LH+14).

3.2.2 Isolation of human luteinised granulosa cells and derivation of fibroblast-like cells

Isolation of luteinised granulosa cells using Percoll gradient centrifugation was carried out as described in section 2.1.3. Twenty-four well cell culture plates were precoated with 25 µl of matrigel (BD Biosciences) and 100,000 viable cells per well were plated in 1ml of serum-free media.

Fibroblast-like cells were obtained from prolonged cultures of follicular aspirates as described in section 2.1.4 and 60,000 cells per well were plated in 1 ml of 10% FBS culture medium on 24-well cell culture plates.

3.2.3 Derivation of fibroblast-like cells from human corpora lutea

Corpora lutea collected during surgery were minced in cell culture conditions and placed in flasks containing 10% fetal bovine serum. Cultures were left to reach confluence and grown until sufficient numbers of cells (60,000 per well) could be obtained for experimental procedures described below.

3.2.4 Primary cell culture treatments

For co-culture experiments, approximately 60,000 fibroblast-like cells were added to the wells containing approximately 100,000 luteinised granulosa cells after culture for 5-7

days in serum-free medium. Each pooled experiment for the following treatments was carried out at least 3 times to avoid biological bias.

3.2.4.1 Treatment of fibroblast-like cells with activin A and inhibin A

Cultures of fibroblast-like cells were plated as above and after 6 hours in serum-free culture, the medium was removed and replaced with medium containing either recombinant activin A (R&D Systems, Inc., Abingdon, UK), recombinant inhibin A (NIBSC, Hertfordshire, UK) or an equivalent amount of 0.1% BSA in carrier as a control. After 24 h the culture medium was collected for subsequent zymography and the cells were used for mRNA extraction.

3.2.4.2 Acute hCG treatments in luteinised granulosa cells and co-culture experiments

Pooled luteinised granulosa cells were cultured for 6-8 days with the medium changed every 2–3 days over the course of the culture period. A maximal stimulating dose of hCG (Serono; 100 ng/ml and control) was added. After 24 h medium and cells were stored for subsequent analysis and mRNA extraction.

After 24 h in serum-free media, co-culture cells had fresh serum-free medium added containing either hCG (100 ng/ml and control) or follistatin (R&D Systems; 500 ng/ml and 0.1% BSA control). Follistatin treatments were within physiological levels (100-600 ng/ml) of that previously reported in human follicular fluid (Khoury *et al.*, 1995). Each control contained the carrier solution equivalent to highest concentration added. After 24 h, mRNA was extracted from co-cultures, and controls consisting of fibroblasts only.

3.2.5 Preparation of cDNA from corpora lutea and cultured cells and primer design

Messenger RNA was batch extracted from both frozen human corpora lutea and cultured cells, and reverse transcribed into cDNA using random hexamers as described in section 2.1.16. Oligonucleotide PCR primers for each gene investigated were designed using Primer3 software (www.genome.wi.mit.edu/cgibin/primer/primer3.www.cgi) from DNA sequences obtained from GenBank (www.ncbi.nlm.nih.gov). Primers were synthesised by MWG AG Biotech and the 5'to 3' sequences used in this study are listed in Table 3.1.

PCR conditions were as follows; incubation 95°C for 5 min, 35 cycles of 95°C for 30 sec, appropriate annealling temperature (see Table 3.1) for 30 sec, and 72°C for 90 sec, followed by 72 °C for 10 min. PCR products were separated and viewed as described in section 2.1.18.2.

Table 3.1 Sequence of primers and annealling temperatures (Tm°C) used for qualitative and quantitative RT-PCR

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Tm (°C)
MMP-2	ATGACAGCTGCACCACTGAG	ATTTGTTGCCCAGGAAAGTG	61
G6PDH	CGGAAACGGTCGTACACTTC	CCGACTGATGGAAGGCATC	62
Inhibin α	CCAGCTGTGAGGACAAGTCA	CTAGCAGGGGCTCAGAGCTA	56
βA	AGACGCTGCACTTCGAGATT	CCCTTTAAGCCCACTTCCTC	56
Follistatin	CAGTAAGTCGGATGAGCCTGTCT	CCTGGTCTTCATCTTCCTCCTCCT	69
β-glycan	CTGTTCACCCGACCTGAAAT	CGTCAGGAGGCACACACT TA	67
ActRIIA	TTTCCGGAGATGGAAGTCAC	GTCCTGGGTCTTGAGTTGGA	54
Alk 2	AAGGCAGGTATGGTGAGGTG	ACCACAGCTGGGTACTGGAG	55
Alk 4	GAGATTGTGGGCACTCAAGGG	AGCTGGGACAGAGAGTCTTCTTG	60
Smad 2	CTGGGATGGAAGAAGTCAGC	CAGTCCCCAAATTTCAGAGC	55
Smad 3	TGAGGCTGTCTACCAGTTGACC	CCGCTGTTCCAGTGTGTCTTAG	57
Smad 4	GATTGCAGACCCACAACCTT	CTAGGAGCAAGGCAGCAAAC	54

3.2.6 Quantitative analysis of gene expression by RT-PCR

Each assay was optimised using PCR amplification of human placental cDNA. The assays were optimised for $MgCl_2$ concentrations and annealling temperatures. PCR amplifications were performed using Thermostart Taq (AB Gene) in a DNA Engine gradient cycler (MJ Research, Inc.,Watertown, MA) as described in section 2.1.19.1. Data were normalised according to the expression level of G6PDH, determined in duplicate by reference to a serial dilution calibration curve generated for each sample using the standard LightCycler software.

3.2.7 Gelatin zymography

Gelatin zymography was performed on the culture medium from activin A and inhibin A treated fibroblast-like cell cultures as described in section 2.1.11.

3.2.8 Measurement of inhibin A and activin A

Inhibin concentrations in culture medium collected from luteinised granulosa cells were measured using a plate modification of a standard in-house inhibin A radioimmunoassay kindly performed by Ian Swanson (MRC, HRSU Edinburgh). The sensitivity of this assay was 2 pg/ml and was carried out as previously described (Groome *et al.*, 1994). Activin A concentrations were measured using a two-site ELISA kit that measured total activin A (Knight *et al.*, 1996) (Oxford Bio-Innovation, Oxfordshire, UK), following the manufacturer's instructions. This assay had a sensitivity of <78 pg/ml and inter and intra-plate coefficients of variation of <10%.

3.2.9 Immunohistochemistry

Immunolocalisation was carried out using antibodies recognising phosphorylated Smad 2/3 (New England Biolabs, Hertfordshire, UK), activin βA subunit (concentration 4.3μg/ml, kindly provided by Prof. N. Groome, Oxford Brookes University, Oxford, UK), ActRIIA and ALK4 (kindly provided by R. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden) in 5 μm paraffin tissue sections of human corpora lutea prepared on poly-L-lysine-coated microscope slides. These sections were dewaxed, rehydrated and washed in TBS/0.1% Tween-20 and TBS respectively. Sections for phosphorylated Smad 2/3 were subjected to antigen retrieval as described in section 2.1.9.3. All sections were washed and placed in 3% H_2O_2/methanol as described in section 2.1.9.4.1.

As described in section 2.1.9.4.3 normal goat serum (NGS) diluted 1:5 in TBS containing 5% BSA (NGS/TBS/BSA) was added to phosphorylated Smad 2/3, ActRIIA and ALK4 sections, while βA sections were blocked in normal rabbit serum (NRS, Diagnostics Scotland) 1:5 in TBS for 1 h at room temperature. Primary antibodies were diluted in respective blocking solutions and incubated on sections overnight at 4°C (phosphorylated

Smad 2/3; 1:1000, βA; 1:500, ActRIIA; 1:400, ALK4; 1:400). Sections were washed and incubated with secondary antibodies [phosphorylated Smad 2/3, ActRIIA and ALK4; biotinylated goat antirabbit IgG diluted 1:500 in NGS/TBS/BSA. βA; rabbit antimouse diluted 1:25 in TBS (DAKO)] for 1 h.

Phosphorylated smad 2/3, ActRIIA and ALK4 sections were incubated in avidin-biotin complex-HRP (DAKO) for 1 h, and βA sections were incubated in mouse peroxidase anti-peroxidase (PAP; DAKO) diluted 1:100 in PBS. Incubations were at room temperature for 1 h and all sections were washed in TBS (2 x 5 min) and bound antibodies visualised by incubation with liquid DAB (DAKO) as described in section 2.1.9.7. Sections were counterstained lightly with hematoxylin to enable cell identification. Negative controls for each antibody examined were performed identically to the above protocol with primary antibody incubations substituted with blocking serum.

3.2.10 Double fluorescent immunohistochemistry

Sections were washed, subjected to antigen retrieval, and blocked as described above. Negative controls were performed as above. Washes detailed below were for 5 min each, and incubations were at room temperature unless otherwise specified. Rabbit anti-phosphorylated Smad 2/3 was diluted 1 in 100 in NGS/TBS/BSA and incubated on sections overnight at 4°C. Sections were washed, incubated with goat anti-rabbit IgG 488 (DAKO) diluted 1 in 200 in PBS for 1 h then washed in PBS. Sections were re-blocked with NGS/TBS/BSA for 1 h then incubated with mouse anti-CD31 or mouse anti-CD68 (DAKO) diluted 1 in 20 in NGS/TBS/BSA overnight at 4°C. Sections were washed, incubated with biotinylated goat antimouse IgG (DAKO) diluted 1 in 500 in NGS/TBS/BSA for 30 min and then washed in TBS.

The fluorochrome streptavidin 546 Alexafluor (Molecular Probes) diluted 1 in 200 in PBS was incubated on slides for 1 h. For the labelling of nucleic acids, sections were counterstained with a nuclear-specific blue fluorescent label (To-Pro 3) as described in section 2.1.10.3.

3.2.11 Statistical analysis

Statistical analyses were carried out, after confirmation of normal distributions for parametric analysis, using a paired t-test when treatment and control samples were analysed or with an analysis of varience (ANOVA) when more than two treatments were analysed. Where significant differences were observed ($p<0.05$) using ANOVA, pairwise comparisons were carried out using Bonferroni's Multiple Comparisons Test. All statistical tests are highlighted in the Figure legends and differences are given.

3.3 Results

3.3.1 MMP-2 and MMP-9 activity in primary cell culture

Matrix metalloproteinase activity was examined in primary cell cultures of luteinised granulosa cells and fibroblast-like cells using gelatin zymography (Figure 6.1). Primary cultures of luteinised granulosa cells secreted MMP-9 whilst little MMP-2 activity was detected. Conversely, and as anticipated, fibroblast-like cells secreted large amounts of MMP-2. Co-cultures of luteinised granulosa cells and fibroblast-like cells secreted both zymogens.

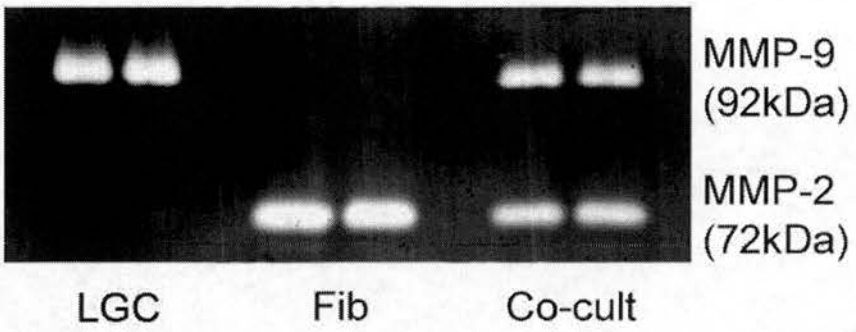


Figure 3.1 Representative gelatin zymogram of primary and co-cultures of luteinised granulosa cells and fibroblast-like cells. Luteinised granulosa cells (LGC) secreted MMP-9 and very little MMP-2. Fibroblast-like cells (Fib) secreted only MMP-2 whilst co-cultures (co-cult) of the two cells types show activity for both zymogens.

3.3.2 The effect of activin A on the expression of MMP-2 in fibroblast-like cells

MMP-2 activity in primary cell cultures was increased by activin A in a dose dependant manner (10 – 100 ng/ml, $p<0.05$, $r2=0.55$, by linear regression). As intrafollicular concentrations of activin A have been reported to be in the range of 0.42-26.3 ng/ml (Fujiwara *et al.*, 2000) the response to physiological concentrations of activin A (10 ng/ml, 25 ng/ml) were investigated in detail (Figure 3.2). Both MMP-2 expression ($p<0.05$, by ANOVA) and activity ($p<0.05$, ANOVA) were increased when compared to controls when fibroblast-like cells were exposed to 25 ng/ml for 24 h (Figure 3.2).

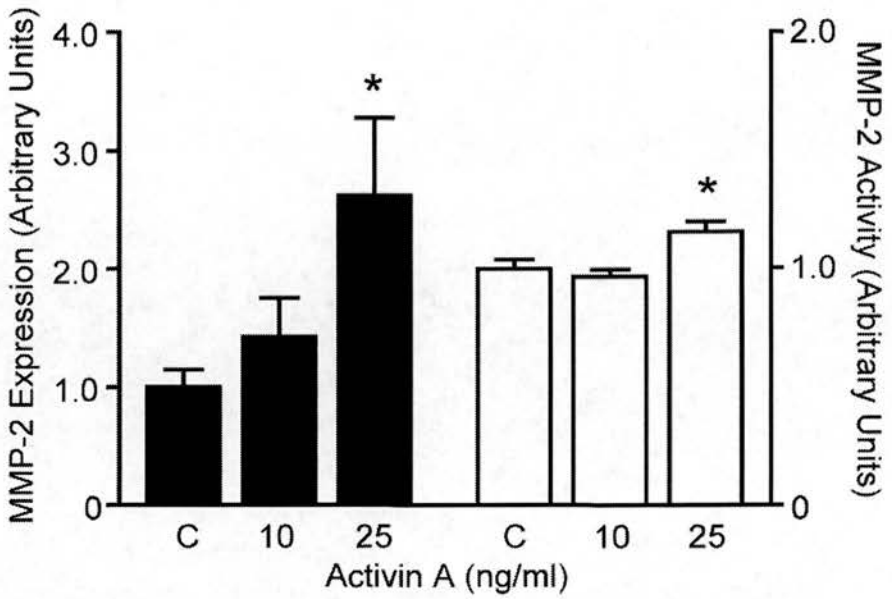


Figure 3.2 Activin A increases MMP-2 in primary cultures of fibroblast-like cells. Relative MMP-2 mRNA expression, using real-time quantitative RT-PCR, is shown in solid bars and MMP-2 activity, using gelatin zymography, is shown in open bars. MMP-2 expression ($p<0.05$, ANOVA) and activity ($p<0.05$, ANOVA) was significantly increased in fibroblast-like cells exposed to 25 ng/ml activin A in culture.

Correspondingly the primary cultures of fibroblasts expressed activin receptors (I and II) and Smad 2, 3 and 4 as well as MMP-2 (Figure 3.3).

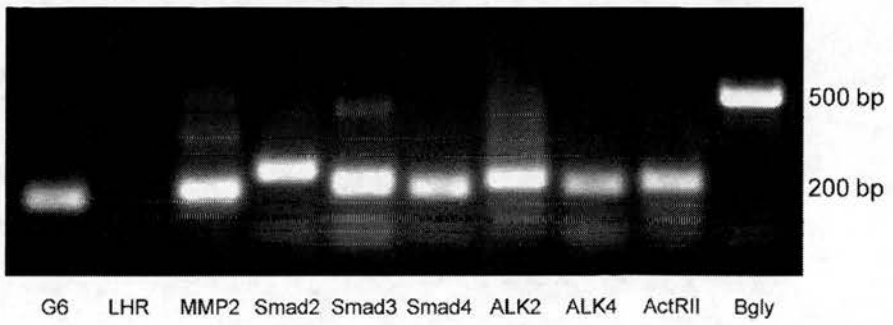


Figure 3.3 Fibroblast-like cells show mRNA expression for housekeeping gene G6PDH and MMP-2, however they do not express the LHR, indicative the activin action via hCG mechanisms is under paracrine control. Furthermore the expression of activin receptors and signalling pathways can be confirmed in these cells. Bands represent 200 and 500 bp as indicated. G6=G6PDH, LHR=LH receptor, ALK2=activin like-kinase 2, ALK4=activin like-kinase 4, ActRII=activin receptor II, Bgly=β-glycan.

It is believed that the effect of activin A on fibroblast-like cells is specific as cells exposed to inhibin A show no change in MMP-2 production ($p>0.05$, ANOVA) as evident in Figure 3.4.

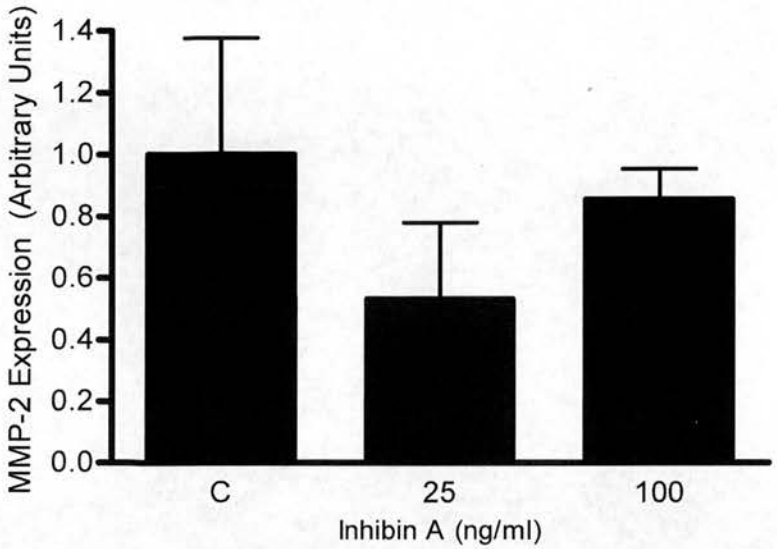


Figure 3.4 Addition of Inhibin A at various concentrations had no effect upon MMP-2 expression in primary cultures of fibroblast-like cells ($p>0.05$, ANOVA).

Furthermore, pilot studies (n=2 corpora lutea) on fibroblast-like cells obtained from prolonged cultures of disaggregated human corpora lutea exposed to 25 ng/ml activin A also demonstrated the same effects on MMP-2 expression (Figure 3.5). It is therefore likely that activin A could act as a regulator of luteal fibroblast MMP-2 production at physiological concentrations.

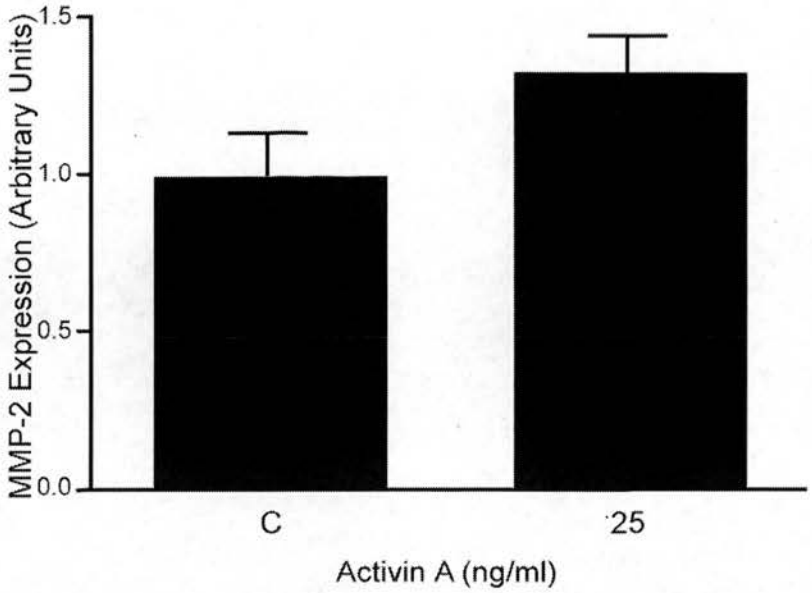


Figure 3.5 Pilot studies using fibroblast-like cells derived from disaggregating human corpora lutea (n=2) demonstrate the same effect for MMP-2 expression to increase in the presence of 25 ng/ml of activin A.

3.3.3 Identification of activin synthesis and action in human corpora lutea

Corpora lutea, at each stage of the luteal phase, have the potential to synthesize activin A, respond to activin A and inhibit its action as they express mRNA for βA subunit, activin receptors (I and II), Smad 2 and 3 and the common Smad 4 as well as inhibin α subunit, betaglycan and follistatin (data not shown). The activin βA subunit is localised to the LH-responsive steroidogenic cells of corpora lutea at each stage of the luteal phase (Figure 3.6A). Although activin receptors can be found on luteal steroidogenic cells, it is notable that luteal fibroblasts, that secrete MMP-2, express both activin receptors I and II (Figure 3.6B,C) and nuclear phosphorylated Smad 2/3 (Figure 3.6D) at each stage of the luteal phase.

3.3.4 Change in Smad signalling across the luteal phase

We investigated phosphorylated Smad 2/3 across the luteal phase by immunohistochemistry (Figure 3.6D-H). Nuclear phosphorylated Smad 2/3 could be detected at all stages of the luteal phase in both the steroidogenic (granulosa lutein cells and theca lutein cells (Figure 3.6D)) and stromal cell compartments. Although it was detected in the fibroblast layer, it was clear that not all cells in this layer immunostained equally. Therefore phosphorylated Smad 2/3 was co-localised with other cell markers. Very little phosphorylated Smad 2/3 immunostaining was noted in endothelial cells (CD31 +ve cells) when compared to neighbouring stromal cells (Figure 3.6) at any stage of the luteal phase, although low level staining could be noted in some cells of vessels (Figure 3.6F). In all sections examined, the fibroblast layer contained macrophages (CD68 +ve cells). Dual immunostaining suggested that there was less phospho-Smad 2/3 immunostaining in the macrophages (Figure 3.6G,H) than the fibroblasts.

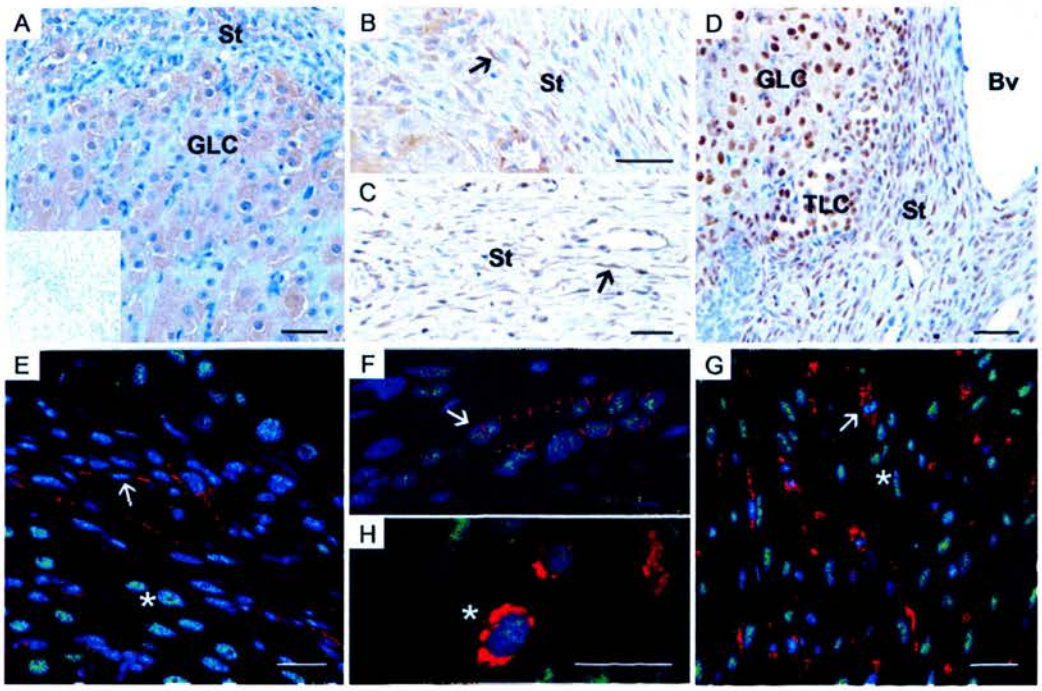


Figure 3.6 Immunolocalisation of factors involved in activin/TGF-β signalling in human corpora lutea. **A,** Light-field of a late-luteal human corpus luteum showing positive brown staining of the βA-subunit in the granulosa-lutein cells (GLC) with little or none observed in the surrounding fibroblast layer. Insert shows no staining in the negative control serial section. **B,** Positive staining for the activin receptors ALK4 and ActRIIA **C,** in the surrounding fibroblast layer of a mid-luteal corpus luteum localised to cells that resemble fibroblast cells (arrows). **D,** Phosphorylated Smad 2/3 is expressed during all stages of the luteal phase. Positive staining for this receptor activated Smad is evident in the nucleus of granulosa-lutein cells (GLC), thecal-lutein cells (TLC) and surrounding fibroblast layer (F) of the corpus luteum with little staining around blood vessels (Bv). **E,** Double immunofluorescence of endothelial cells (CD 31) (arrow) in red co-localised with phosphorylated Smad 2/3 (green) in a mid-luteal corpus luteum. Nuclear phosphorylated Smad staining is clear in presumptive fibroblast cells (asterisk) located in the stromal area of the corpus luteum and less marked in the endothelial cells (arrow). Nuclear staining is depicted in blue. **F,** High power images illustrate a low level of phosphorylated Smad 2/3 (green) in some endothelial cells (arrow) in red. **G,** Low power image of phosphorylated Smad 2/3 (green) and macrophages (CD 68 +ve cells) (red) in a late-luteal corpus luteum. Phosphorylated Smad 2/3 is localised to fibroblast cells (asterisk) and less so in the macrophages (arrow) stained in red. **H,** Higher power view of an individual macrophage (red) in the same tissue section (asterix) showing low levels of phospho-Smad (green). Scale bars A-D, 40 μm E-H, 20 μm.

3.3.5 Quantification of Smad signalling across the luteal phase

In order to investigate changes in Smad signalling across the luteal phase the expression of Smad 2 and Smad 3 was investigated using quantitative RT-PCR (Figure 3.7) in carefully dated corpora lutea. Smad 3 expression increased to a maximum in the late-luteal phase and was different between early and mid ($p<0.05$, ANOVA), and early and late ($p<0.01$, ANOVA) (Fig. 3.7). The expression of Smad 2 mRNA (Figure 3.7A) was also assessed over the luteal phase, and although it demonstrated a similar trend to that of Smad 3, the differences did not reach significance.

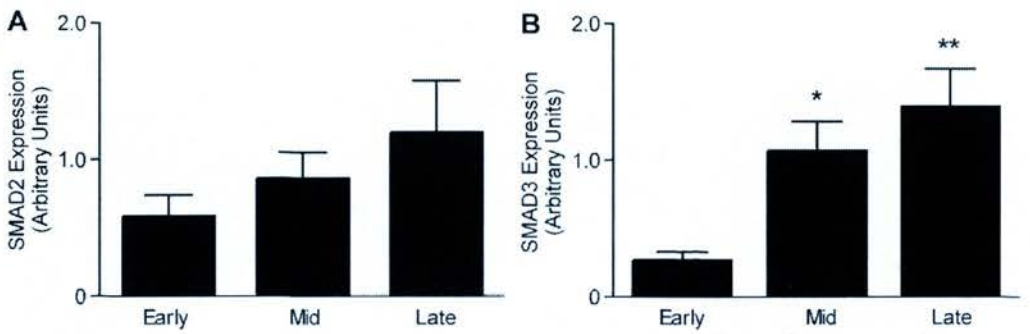


Figure 3.7 Expression of the receptor activated Smads in human corpora lutea over the luteal cycle. **A,** Real-time quantitative RT-PCR demonstrated that the mRNA expression of Smad 2 increased over the luteal phase (n=6 for each group), however a non-significant trend ($p>0.05$, ANOVA) was observed. **B,** The expression of Smad 3 was significantly increased from the early luteal phase in both mid luteal ($p<0.05$, ANOVA) and late-luteal ($p<0.01$, ANOVA) samples.

3.3.6 Effect of hCG on activin A and its inhibitors in primary cell cultures of steroidogenic cells

To test the hypothesis that during maternal recognition of pregnancy the exposure of hCG to the corpus luteum results in less activin A signalling from the steroidogenic cells the effect of hCG in primary cultures of luteinised granulosa cells was assessed. The secretion of both inhibin A (range 10-500 pg/ml) ($p<0.05$, t-test) and activin A (range 1-5 ng/ml) ($p<0.05$, t-test) was increased by the addition of hCG within 24 h (Figure 3.8). Experimental results were standardised to their controls to consider variation within individual experiments.

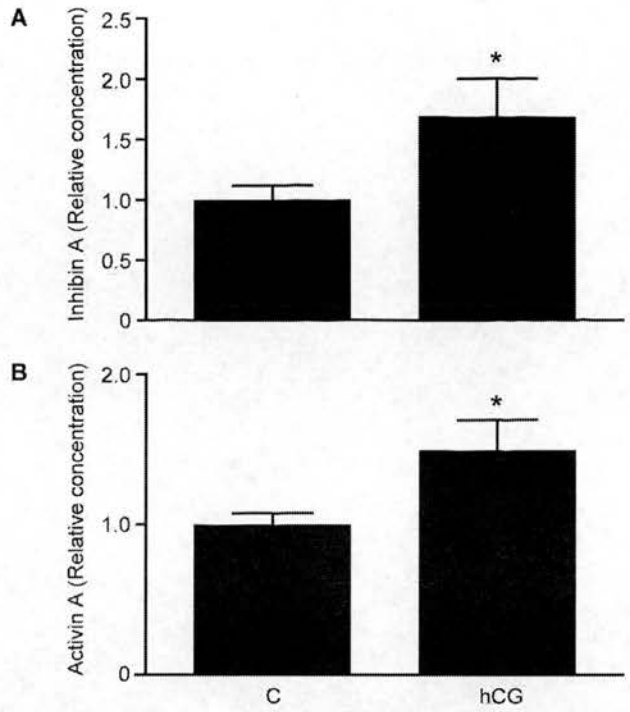


Figure 3.8 The effect of hCG on elements of the activin pathway in primary cell cultures. **A,** Luteinised granulosa cells show an increase in inhibin A ($p<0.05$, t-test) and **B,** activin A ($p<0.05$, t-test) production when treated with hCG (100 ng/ml) for 24 h relative to control samples.

Although exposure to hCG did not change the inhibin:activin ratio, luteinised granulosa cells markedly increased their expression of the activin-binding protein, follistatin ($p<0.01$, t-test) in response to hCG stimulation (Figure 3.9). In the presence of hCG, the up-regulation of follistatin may therefore be able to reduce the local actions of activin A.

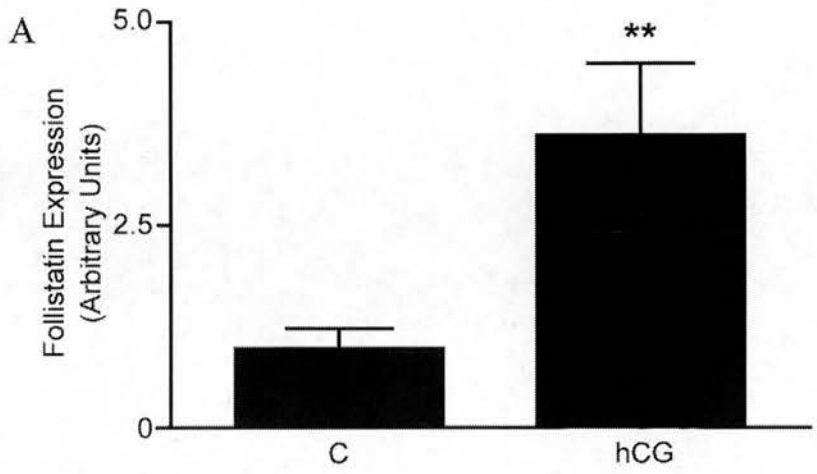


Figure 3.9 The effect of hCG on the expression of the activin binding protein, follistatin ($p<0.01$, t-test) in primary cultures of luteinised granulosa cells.

3.3.7 Evidence that luteal MMP-2 is under the paracrine control of activin A

To test the hypothesis that hCG could inhibit MMP-2 expression in a paracrine signalling fashion, a novel primary cell co-culture system of luteinised granulosa cells and fibroblast-like cells was employed (Duncan *et al.*, 2005b). In these co-cultures the primary source of MMP-2 in the culture medium are the fibroblast-like cells (Figure 3.1). The addition of hCG to fibroblast-like cell cultures had no effect on the expression of MMP-2 ($p>0.05$, t-test) (Figure 3.10). However, co-cultures exposed to hCG demonstrated a significant reduction in the expression of MMP-2 ($p<0.05$, t-test) (Figure 3.10), suggestive that hCG is acting on luteinised granulosa cells to influence fibroblast-like cell MMP-2 expression in a paracrine manner.

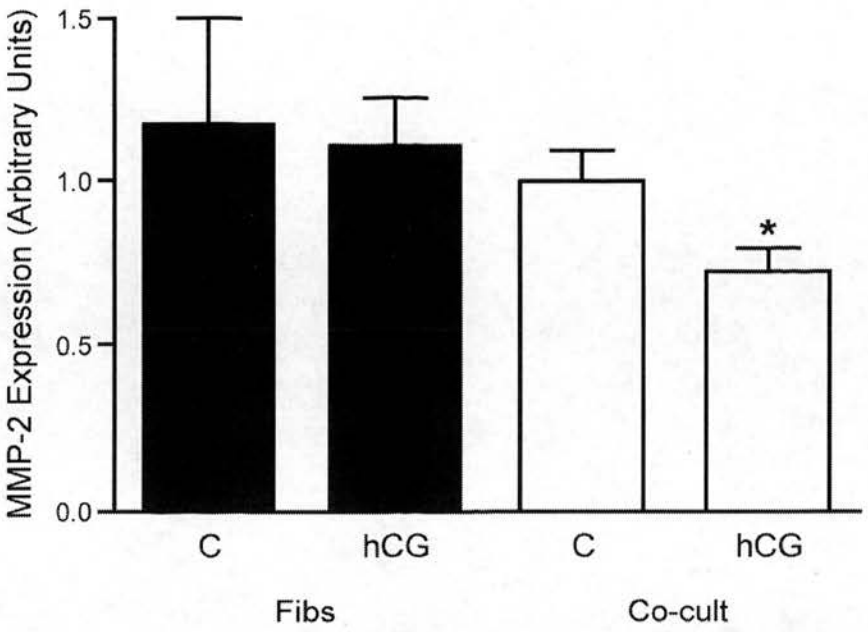


Figure 3.10 *In vitro* model of paracrine signalling in co-culture experiments of luteinised granulosa cells and luteal fibroblast-like cells demonstrates that MMP-2 expression was significantly decreased ($p<0.05$, t-test) in primary co-cultures exposed to hCG (100 ng/ml) for 24 h.

To test the hypothesis that hCG is manipulating MMP-2 expression through a reduction in activin A activity, secondary to an up-regulation of follistatin expression, physiological concentrations of follistatin were added to the primary cell cultures. The addition of follistatin to cultures of fibroblast-like cells had no significant effect upon MMP-2 expression ($p>0.05$, t-test) (Figure 3.11). However follistatin had the same effect as hCG in reducing the expression of MMP-2 in co-cultures of luteinised granulosa cells and fibroblast-like cells (Figure 3.11) ($p<0.01$, t-test).

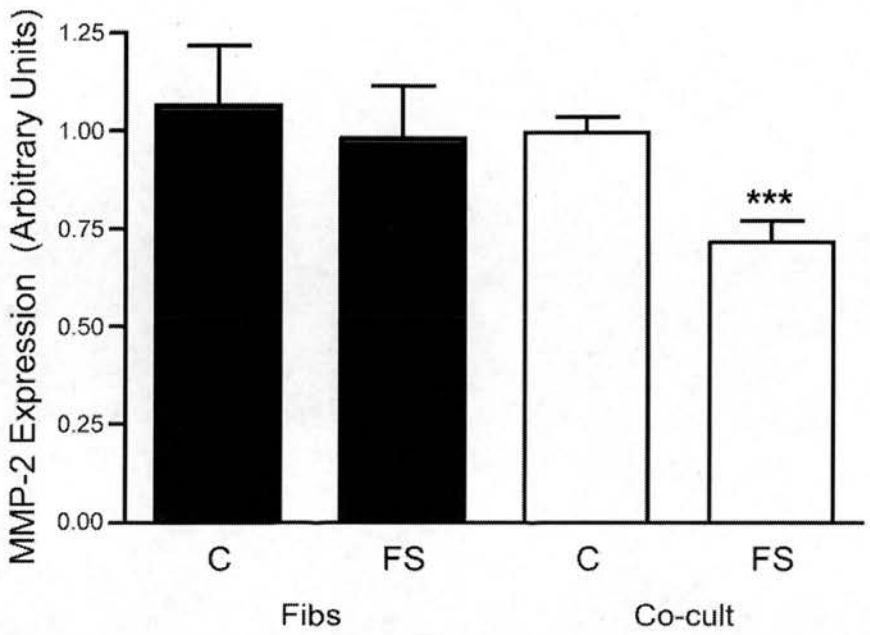


Figure 3.11 Similar to Figure 3.10, the expression of MMP-2 was significantly reduced ($p<0.01$, t-test) in co-cultures treated with follistatin (500 ng/ml) for 24 h. Fibroblast-like cell cultures in both treatment groups were unaffected by either hCG or follistatin ($p>0.05$, t-test).

3.4 Discussion

How luteolysis occurs in women and how it is inhibited by hCG during maternal recognition of pregnancy is not yet understood. What is clear however is that the inhibition of luteolysis by hCG involves disparate effects on cell-types in the corpus luteum that do not express LH/hCG receptors. Previously it has been shown that hCG regulates luteal fibroblast (Duncan *et al.*, 2005b; Duncan *et al.*, 1998a), macrophage (Duncan *et al.*, 1998b) and endothelial cell (Wulff *et al.*, 2001) function. This means that paracrine signalling molecules from steroidogenic cells have key roles in the local regulation of luteal cell function. To date, the identity of these molecules has remained largely elusive. This study shows for the first time, using a combination of observational studies on human corpora lutea combined with interventional studies using human primary cell culture and co-culture models, that activin A is an excellent candidate molecule for a paracrine regulator of luteal remodelling during luteolysis whose action can be inhibited by hCG during maternal recognition of pregnancy.

It has to be highlighted that these studies used luteinised granulosa cells and fibroblast-like cells from the luteinising follicle. This co-culture model allows information about the paracrine interactions that occur during luteolysis. It is possible that dispersed cells from human corpora lutea may respond differently at different stages of the luteal phase. Unfortunately such comprehensive studies using fresh human luteal tissue are almost impossible and it is likely that analysis of activin effects on remodelling of luteal cells will need further assessment in sub-human species.

Activins belong to the structurally related TGF-β superfamily that includes inhibins and BMPs. Members of this family have been shown to have important paracrine regulatory roles in diverse physiological processes (Massague, 1998). Indeed, activin signalling has been shown to be essential in inflammation (Jones *et al.*, 2004), cell proliferation and apoptosis (Hully *et al.*, 1994), fetal development (Matzuk *et al.*, 1995) and male reproduction (de Kretser *et al.*, 2002; Risbridger and Cancilla, 2000). In particular, it has been established that activins may have a paracrine role during the normal ovarian cycle (de Kretser *et al.*, 2002; Welt *et al.*, 2002). Activin can stimulate the proliferation of granulosa cells in small follicles (Miro and Hillier, 1996) and enhance their expression of FSH receptors and aromatase (de Kretser *et al.*, 2002; Xiao *et al.*, 1992). It appears that one of the roles of activin in the ovary is to stimulate smaller follicles and inhibit

luteinisation of larger follicles to maintain the follicle in an FSH-responsive state (de Kretser *et al.*, 2002; Knight and Glister, 2006).

There is evidence to support a paracrine role for activin in the corpus luteum. In the first instance, the corpus luteum has the mechanism to synthesise and secrete activin. Studies in women (Roberts *et al.*, 1993) and primates (Fraser *et al.*, 1993) have localised inhibin α and βA subunit mRNA and protein to the steroidogenic cells of the corpus luteum. Dispersed luteal cells *in vitro* and the intact corpus luteum *in vivo* secrete inhibin A in a regulated manner (Illingworth *et al.*, 1996). The same is seen in cultures of human luteinised granulosa cells. In addition, these cells secrete activin A and activin A is found in the follicular fluid at the time of ovulation (Fujiwara *et al.*, 2000). Indeed, circulating activin concentrations change across the ovarian cycle, with an increase towards the end of the luteal phase (Muttukrishna *et al.*, 1996). As well as expressing the βA subunit mRNA and protein, it is likely that the granulosa-lutein cells of the corpus luteum secrete activin A during the luteal phase.

In addition, this study demonstrates that the corpus luteum has the molecular mechanisms to respond to locally produced activins. Activins signal through two types of transmembrane serine/threonine kinase receptor interactions (Chen *et al.*, 1998) and the cytoplasmic to nuclear translocation of the intracellular Smad proteins (Heldin *et al.*, 1997). Human corpora lutea express both the type I (ALK 2/4) and type II (A) activin receptors as well as components of the Smad (2, 3 and 4) signalling pathway that are induced by activin. Correspondingly, in the present study the receptors and activated nuclear phosphorylated Smad 2/3 have been localised to both steroidogenic and stromal cells of the human corpus luteum. Although the nature of the ligand signalling through the Smad 2/3 pathway is not entirely clear, as TGF-β signals through similar Smads, (Chen *et al.*, 1998; Pangas and Matzuk, 2004) it is likely that the activin signalling cascade is active and activins do act locally on different cells types in the corpus luteum.

Activin action however is highly controlled in physiological systems. It is tightly regulated by various inhibitors at the ligand, receptor and post-receptor levels (Harrison *et al.*, 2005). Follistatin, a local regulator of activin, controls activin signalling by forming biologically inactive complexes with the β-subunits of the activin glycoprotein. Suppression of activin-regulated processes is also achieved by the activin antagonist

inhibin and its co-receptor, betaglycan. Inhibin opposes activin action by competitively binding to the same site of the type II activin receptor. Additionally, inhibins have also been thought to have interactions with specific and high affinity receptors which may then activate signal transduction pathways, such as the inhibitory Smads, that can oppose activin action (Robertson *et al.*, 2000). Betaglycan on the other hand, has a high affinity for inhibin and forms complexes with the type II activin receptor to block the recruitment of the type I receptor (Lewis *et al.*, 2000) that is required to activate the signalling cascade. As well as making activin, the corpus luteum has at least some of the molecular mechanisms required to inhibit activin.

It is not clear whether activin signalling changes across the luteal phase. It is likely there is more activin available to act in the late-luteal phase as the increase in circulating activin A at the time of luteolysis (Muttukrishna *et al.*, 1996) is not seen with regards to its inhibitors, inhibin A (Illingworth *et al.*, 1996; Muttukrishna *et al.*, 1996) and follistatin (Schneyer *et al.*, 2000). Futhermore, immunolocalisation of nuclear phosphorylated Smad 2/3 was evident, which would be detected in the presence of activin signalling, throughout the luteal phase. Although most increases in Smad signalling are through phosphorylation of the proteins, there are also increases in the expression of Smad mRNA in response to ligand activation. Therefore this study investigated the expression of Smad 2 and 3 mRNA across the luteal phase. Expression of both Smads tended to be maximal in the late-luteal phase. These results are compatible with activin action increasing in the lead up to luteolysis.

Both the expression of activin and its local inhibitors are regulated in the corpus luteum. The addition of hCG up-regulates the inhibin α subunit and the secretion of inhibin A from steroidogenic cells *in vitro* and *in vivo* (Illingworth *et al.*, 1996; Muttukrishna *et al.*, 1997). This study hypothesised that the inhibin: activin ratio would increase in the presence of hCG and tested this using luteinised granulosa cells in culture. However, a similar stimulation of both activin A and inhibin A was evident with hCG and consequently no change to the ratio. However, other studies have shown that short-term gonadotrophin stimulation initially increased activin A secretion, but unlike inhibin A, this diminishes later in a time- and dose-dependant manner (Vanttinen *et al.*, 2002) and it is possible that hCG increases the inhibin:activin ratio in the longer-term. In addition, both bound, inactive, activin and free, active, activin were detected by the assay and it is

possible that the ratio of inhibin to active activin changes. This is particularly important as it is clear that follistatin is hormonally regulated. HCG up-regulates follistatin from luteinised granulosa cells in culture and follistatin concentrations in follicular fluid are hormonally regulated (Schneyer *et al.*, 2000). For instance, follistatin is up-regulated in the serum of pregnant women (Khoury *et al.*, 1995). Collectively, these data are suggestive that luteal activin action, although not necessarily secretion, is inhibited by hCG during luteal rescue.

There appears to be a role for increased activin activity during luteolysis. Previously, activin has been shown to inhibit progesterone production by luteal cells *in vitro* (Di Simone *et al.*, 1994), and therefore it may have a role in the changes associated with functional luteolysis. However, this chapter suggests that activin has a role in the remodelling associated with structural luteolysis. One of the key features associated with luteolysis is the up-regulation of MMP-2 in stromal cells (Curry and Osteen, 2003; Duncan *et al.*, 1998a; Endo *et al.*, 1993). Herein it has been shown for the first time that fibroblast-like cell MMP-2 is up-regulated by concentrations of activin (but not inhibin) found inside the human ovary. For clarity, the term fibroblast-like cells and stroma is used to represent the stromal cells that are directly surrounding the steroidogenic cells and invaginating in between them. Furthermore stromal MMP-2 expression can be inhibited by hCG in a paracrine manner both *in vivo* (Duncan *et al.*, 1998a) and in co-culture models *in vitro*. The effect of hCG on MMP-2 in these co-cultures can be replicated by the addition of follistatin at concentrations found within the human ovary. As hCG up-regulates follistatin, these results suggest that activin A may be involved locally in the physiological regulation of MMP-2 activity in the corpus luteum.

MMP-2 is not known for being regulated at the level of its expression. Indeed, an examination of the promoter region of the MMP-2 gene shows less regulatory sequences than other MMPs (Curry and Osteen, 2003), consistent with a gene that is normally constantly expressed. However there are clearly certain circumstances when its expression is regulated. It is increased in fetal membranes during labor (Riley *et al.*, 1999), in the endometrium during menstruation (Salamonsen and Woolley, 1996) and more importantly, it is increased during natural and induced luteolysis in a wide range of different species (Curry and Osteen, 2003; Duncan *et al.*, 1998a; Endo *et al.*, 1993). The factors involved in this regulation are not certain. There has been a suggestion that

steroids are involved in its regulation in the endometrium (Salamonsen and Woolley, 1999) and cytokines such as tumor necrosis factor-α (TNF-α) may be important in the regulation of endometrial fibroblast (Braundmeier and Nowak, 2006) and bovine luteal (Zhang *et al.*, 2005) MMP-2 expression. Extravillious trophoblasts over-expressing Smad 4 demonstrate a clear up-regulation of MMP-2 production, mimicking the effect of TGF-β in this particular system (Lin *et al.*, 2006). The same theory may apply to activin A in fibroblast cells of the corpus luteum as it is known they express the Smad proteins common to both TGF-β and activin signalling and that activin A in fibroblast-like cells increases MMP-2 production. The present study demonstrates that activin A can up-regulate-luteal MMP-2 expression and activity *in vitro* at physiological concentrations.

There is some evidence, in other systems, that activin may well regulate MMP-2 expression. Activin A has been found to regulate MMP-2 in both mouse peritoneal macrophages (Ogawa *et al.*, 2000) and human decidua (Caniggia *et al.*, 1997; Jones *et al.*, 2006). In addition it has been suggested that activin can up-regulate both MMP-9 and MMP-2 expression in cultured human cytotrophoblast cells (Caniggia *et al.*, 1997). However this was thought to be secondary to activin affecting the differentiation status of these cells. Another molecule from luteal fibroblasts with a role in tissue remodelling during luteolysis is CTGF (Duncan *et al.*, 2005b). Previous studies have shown that activin also up-regulates its expression in luteal fibroblast-like cells (Duncan *et al.*, 2005b), although this does not appear to be the only regulating factor. It is not clear whether other molecules as well as activin can regulate-luteal MMP-2 expression during luteolysis.

If activin A is involved in regulating tissue remodelling during luteolysis it is likely to be self-limiting in nature. This is because the cellular source of activin is the steroidogenic cells that undergo programmed cell death when the corpus luteum is cleared from the ovary (Behrman *et al.*, 1993; Fraser *et al.*, 1999). It is difficult to determine if observations made in non-primate species are relevant to women as there are major species differences in the regulation of the corpus luteum. In the mouse, where ovarian activin βA expression has been conditionally knocked out (Roy and Matzuk, 2006), the ovary contains multiple corpora lutea when examined. While is it tempting to speculate that luteolysis may be affected, it has to be remembered that inhibin A will also be

knocked out and this may well result in higher FSH concentrations would be expected to cause more ovulations.

Observational studies on human corpora lutea inform us that the steroidogenic cells expressing LH/hCG receptors can secrete activin A and the stromal cells that express MMP-2 have the molecular machinery to respond to locally produced activin. Such observations however do not tell us the roles of activin and interventional studies are required. It is now not practical to collect enough well characterised human corpora lutea to disaggregate the cells and manipulate these cells in culture and other model systems have to be used. The culture of luteinised granulosa cells and the derivation of fibroblast-like cells have the potential to replicate the luteal steroidogenic cell/stromal cell interactions *in vitro*. Although luteinised granulosa cells are LH/hCG-responsive and function in primary culture for 14 days, they may differ from mature granulosa-lutein cells. In addition, although the phenotype of the fibroblast-like cells is identical to luteal stromal cells in all the genes so far analysed, (Duncan *et al.*, 2005b) and expression patterns of MMP-2 from prolonged cultures of fibroblasts obtained from corpora lutea, there may be differences. Indeed, previous studies have shown that there are at least two types of fibroblast in the human corpus luteum (Maybin and Duncan, 2004). Furthermore, in culture, the cells do not have the local interaction with immune cells and endothelial cells seen *in vivo*.

Whilst it must be accepted that there are caveats to this model system and indeed, the *in vitro* cell culture and co-culture models may not entirely mimic what is happening within the corpus luteum, such model systems can give valuable insights into mechanistic functions that otherwise may not be dissected. Therefore care needs to be used in the interpretation of their findings and used in conjunction with observational studies, this study has used the most appropriate model to study and manipulate cell-cell interactions in the human corpus luteum.

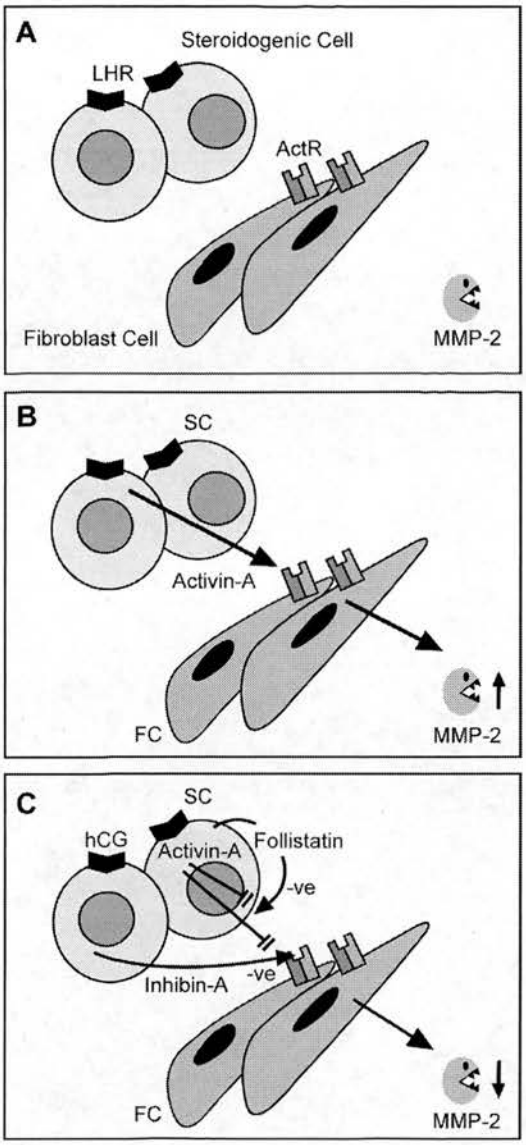
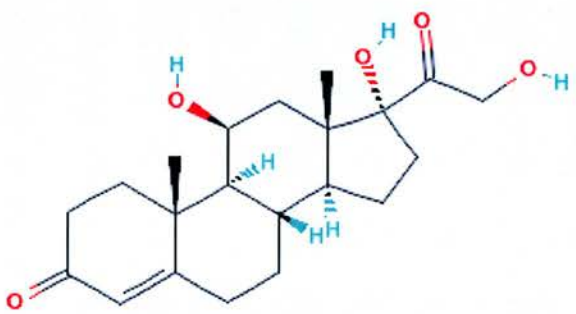


Figure 3.12 Schematic of the model proposed for the paracrine regulation of MMP-2 expression by hCG in human luteal cells. **A,** Steroidogenic cells (SC), but not fibroblast-like cells (FC) express the LH/hCG receptor. The fibroblast cells secrete MMP-2 and express activin receptors. **B,** During the late-luteal phase increasing activin A action from the steroidogenic cells up-regulates the expression of MMP-2 from the fibroblast cells. **C,** In the presence of hCG during maternal recognition of pregnancy, the increase of inhibin A will block activin binding to its receptors while follistatin will bind and biologically inactivate secreted activin A, resulting in decreased activin signalling thus preventing the increased expression of MMP-2.

In conclusion, this chapter postulates that activin A may have a physiological role in luteolysis and part of this role is to stimulate-luteal MMP-2 expression. The physiological role of hCG may therefore serve to impede activin action (Figure 3.12) and this will facilitate luteal maintenance by inhibiting luteolysis and allowing the maternal recognition of pregnancy.

4 Role of luteal glucocorticoid metabolism during maternal recognition of pregnancy in women



One of the major enigmas in human luteal physiology is the identification of luteotrophic and luteolytic factors. Evidence from Chapter 3 clearly suggests that activin A is an excellent candidate molecule responsible for the tissue remodelling associated with luteolysis. However luteotrophic paracrine factors still remain elusive. Consequently, the major focus of this chapter was to investigate a role for cortisol as a potential luteotrophin in the human corpus luteum.

(Structure of cortisol from http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=148533)

4.1 Introduction

The human corpus luteum develops from the post-ovulatory follicle to become one of the most active endocrine glands in the body. During the luteal life-span this remarkable structure will experience very high cell turnover, intense angiogenesis and considerable steroid production within a 14-day period. Such highly organised events rely upon stringent paracrine interactions between neighbouring and surrounding cells. In a non-conception cycle the corpus luteum will undergo luteolysis, a complex process whereby the tissue is subjected to a loss of structural and functional integrity that is associated with an immune cell influx (Duncan *et al.*, 1998b), increase in matrix metalloproteinases (Duncan *et al.*, 1998a), cell death (Fraser *et al.*, 1999) and vascular regression (Fraser and Duncan, 2005). Remarkably however, such dramatic remodelling and rapid tissue removal occurs in an orderly fashion without any evidence of permanent scar tissue.

Within the ovary, ovulation and the resulting folliculo-luteal transition has been likened to inflammatory response, as a result of the acute hemodynamic, cellular and biochemical changes that occur at the site of follicle rupture (Espey, 1980; Hillier and Tetsuka, 1998). Key studies have clearly shown that the repetitive damage from consecutive ovulations must be quickly repaired in anticipation for the next ovulatory cycle, and locally produced glucocorticoids are involved (Gubbay *et al.*, 2005; Rae *et al.*, 2004a; Yong *et al.*, 2002). Cortisol, the most important glucocorticoid, is well known to minimise inflammatory damage to tissues by encouraging wound healing and subsequent repair. Clear evidence of this phenomenon is apparent from the switch of 11βHSD1 isoforms over the ovarian cycle, complemented by the increased concentrations of cortisol in follicular fluid following the LH surge (Harlow *et al.*, 1997). At present, both the presence of 11βHSD1 (that tends to generate cortisol) and 11βHSD2 (that tends to inactivate cortisol) isoforms, and their activities, have been reported in the ovaries of several species (Michael *et al.*, 2003).

Throughout the human body, glucocorticoids are well known to exhibit a plethora of physiological roles. Whilst most of these actions have been best characterised in body systems such as kidney, liver, adrenal and local inflammatory responses, influences on human fertility, oocyte maturation and the establishment of a functional corpus luteum have been suggested (Andersen, 2002; Fateh *et al.*, 1989; Jimena *et al.*, 1992; Michael and Cooke, 1994). It has also been reported that as glucocorticoids can inhibit

endothelial cell proliferation (Gaytan *et al.*, 2002), they may have a role in the local regulation of angiogenesis.

With the knowledge that glucocorticoids are involved in inflammatory-associated events in the ovary and may adversely affect angiogenesis, it was hypothesised that local cortisol action may have a role in the luteolytic process. Although governed by different luteolytic mechanisms than in women, elegant studies in the rat have however identified a potential role for 11βHSD2 in the regressing corpus luteum (Waddell *et al.*, 1996). Therefore, based upon the findings in the rat study, coupled with much evidence for glucocorticoids in tissue and scar regeneration, this study hypothesised that glucocorticoids were involved across the luteal lifespan.

4.1.1 Aims

To localise and identify a role for glucocorticoid metabolism in the human corpus luteum

To investigate luteal glucocorticoid reception in the human corpus luteum

To establish the role of hCG upon the regulation of local glucocorticoids (via the 11βHSD enzymes and the glucocorticoid receptor) in primary cultures of luteinised granulosa cells

To establish whether changes in cortisol synthesis and metabolism might be key events in the regulation of the tissue remodelling associated with luteolysis.

4.2 Materials and Methods

4.2.1 Collection of human corpora lutea

Human corpora lutea were collected from women with regular menstrual cycles undergoing hysterectomy for benign conditions and dated on the basis of the urinary LH surge as fully described in section 2.1.2.2.

4.2.2 Isolation of human luteinised granulosa cells and derivation of fibroblast-like cells

Isolation of luteinised granulosa cells using Percoll gradient centrifugation was carried out as described in section 2.1.3. Twenty-four well cell culture plates were precoated with 25 µl of matrigel (BD Biosciences) and 100,000 viable cells per well were plated in 1ml of serum-free media.

Fibroblast-like cells were obtained from prolonged cultures of follicular aspirates as described in section 2.1.4 and 60,000 cells per well were plated in 1ml of 10% FBS culture medium on 24-well cell culture plates.

4.2.3 Primary cell culture treatments

Each pooled experiment for the following treatments was carried out at least 3 times to avoid biological bias.

4.2.3.1 Assessment of the acute effects of hCG and progesterone

Luteinised granulosa cells plated as above had fresh medium changed every 2-3 days over course of culture and treatment was carried out on day 6 or 7 of culture. The treatments groups were, 1) Controls with LDL, (50 mg/l Sigma), 2) hCG (10 ng/ml, Serono) with LDL, 50 mg/l, and 3) hCG (10 ng/ml) and LDL (50 mg/l) in conjunction with 100 µM aminoglutethimide (Sigma). After 24 h, medium and cells were stored for steroid analysis and mRNA extractions respectively. Progesterone concentrations in the culture media were determined by an in house radioimmunoassay as described in section 4.2.10.

4.2.3.2 Manipulation of hCG in prolonged cultures of luteinised granulosa cells

In order to mimic the luteal phase in primary cell culture, luteinised granulosa cells were plated as described above and grown for 12 days as described in section 2.1.3.1. Briefly, cells were stimulated with low dose hCG (1 ng/ml) with LDL (50 mg/l) beginning on day 2 and this was repeated every 2nd day until day 7 when treatments were replaced with maximal doses of 100 ng/ml hCG/LDL or LDL alone. Cells were analysed after seven days with hCG and on day 12 in the presence or absence of hCG to mimic the progesterone secretion profile of late-luteal and luteal rescue stages respectively (Duncan *et al.*, 2005a).

4.2.3.3 Relative cell counts for various steroid and steroid inhibitor treatments

Pooled luteinised granulosa cells were cultured as above for seven days in the presence of the same carrier ethanol concentrations in each well. The experiments were piloted to determine the appropriate concentrations of reagents and final experiments were repeated three times in triplicate. The treatments were, 1) control, 2) aminoglutethimide (100 μM), 3) RU486 (100 μM; Sigma) 4) RU486 (100 μM) with progesterone (500 μM; Sigma), 5) RU486 (100 μM) with cortisol (500 μM; Sigma) and 6) RU486 (100 μM) with hCG (100 ng/ml). After seven days, the cells were removed by trypsinisation, resuspended and counted using a haemocytometer. Values were taken as the mean of at least four separate counts by an observer blinded to the treatments and related to controls.

4.2.3.4 Treatment of cultured fibroblast-like cells with cortisol

Cultures of fibroblast-like cells were plated as above. After 6 hours in serum-free culture, the medium was removed and replaced with medium containing either cortisol (100 nM) or an equivalent amount of the ethanol carrier as a control. After 24 hours the culture medium was collected for subsequent zymography and the cells were used for mRNA extraction.

4.2.4 Preparation of cDNA from human corpora lutea and cultured cells

Messenger RNA was batch extracted from frozen human corpora lutea and reverse transcribed into cDNA using random hexamers as described in section 2.1.16. Cultured

cell mRNA was extracted using RNeasy mini-spin columns after lysis by the addition of RNeasy lysis buffer (Qiagen). Lysates were frozen until processed as per manufacturers' protocols then DNase treated with on-column DNaseI (Qiagen) and quantified using the NanoDrop ND-1000 Spectrophometer (NanoDrop Technologies). Messenger RNA was then reverse transcribed into cDNA using random hexamers (Applied Biosystems).

4.2.5 Quantitative analysis of gene expression by RT-PCR

Quantitative real-time PCR was carried out on the ABI PRISM 7700 sequence detection system (Applied Biosystems) using specific primers and probes (Eurogentec) for each gene of interest (Table 4.1) and levels were related to a ribosomal 18S internal control (Applied Biosystems). QRT-PCR was performed with an extension temperature of 72°C, and 30 cycles of amplification. All samples were performed in duplicate and a relative comparison was made to an appropriate tissue control, either human placental or liver cDNA.

Table 4.1 List of all primer/probe sequences used for Taqman qRT-PCR. Sequences for 11βHSD1, 11βHSD2 and GRα obtained from Rae et al (Rae *et al.*, 2004a).

Gene	Fwd primer 5'-3'	Rev primer 5'-3'	Probe 5'-FAM-TAMRA-3'
GRα	GCGATGGTCTCAGAAACCAAAC	GCAGAGGATAACTTCCTCTGTAATCTC	TCAGAGCCTCAGCAACCTTCACTGCA
11βHSD1	AAGATGTTCCTGCATGGATTTC	AGCTCTGCGCCAAGAAGAAGT	TGACAGCTCACTCTGGACCACTCTTCTGA
11βHSD2	GGCCAAGGTTTCCCAGTGA	GTTGTGCCAGGAGGGTGTTT	CTCTGCGCCTCTCCACTGTTTCATGA
MMP-2	TTCCTGGGCAACAAATATGAGA	TGGTCGCACACCACATCTTT	AGCGCCGGCCGCAGTGA (Jordan *et al.*, 2004)

4.2.6 Histology

Masson's modified trichrome staining was carried out on 5 μm paraffin sections of human corpora lutea. Briefly, sections were dewaxed, rehydrated in alcohol and washed in water. Sections were then placed in Celestine blue for 3 min, washed, placed in Mayer's acid haematoxylin solution for 3 min, washed and then counter-stained with Biebrich scarlet-acid fuchsin solution for 15 min. After washing the sections were

differentiated in 5% phosphotungstic acid for 15 min before being placed directly into aniline blue solution for 5-10 min, washed, dehydrated and mounted for microscopy.

4.2.7 Immunohistochemistry

Immunolocalisation of GR was carried out using a rabbit polyclonal antibody (ABR, Cambridge BioScience, UK) in 5 μm paraffin tissue sections of human corpora lutea prepared on poly-L-lysine-coated microscope slides. These sections were dewaxed, rehydrated, washed in PBS, subjected to antigen retrieval as described under section 2.1.9. All sections were washed and blocked in 3% H_2O_2/methanol, avidin/biotin and normal goat serum as described in section 2.1.9.

GR antibody was diluted 1:2000 in blocking solution and incubated on sections overnight at 4°C. After rinsing, slides were incubated with the biotinylated goat anti-rabbit immunoglobulin G (IgG; diluted 1:500 in NGS/TBS/BSA) secondary antibody (DAKO) for 1 h. After washing, the sections were incubated in ABC-HRP (DAKO) and binding was visualised by incubation with liquid DAB (DAKO). Sections were counterstained lightly with hematoxylin to enable cell identification. Negative controls were performed identically to the above protocol with primary antibody incubations substituted with blocking serum.

4.2.8 Fluorescent immunohistochemistry

Sections were washed, subjected to antigen retrieval, and blocked as described above and negative controls were performed as above. Rabbit anti-GR and rabbit anti-11βHSD1 (Cayman/IDS Ltd, Bolton UK) diluted 1 in 100 in NGS/PBS/BSA were incubated on sections overnight at 4°C. Sections were washed and slides were incubated with goat anti-rabbit IgG 488 (DAKO) diluted 1 in 200 in PBS for 1 h.

Sections that were labelled with anti-GR were subjected to further co-localisation experiments. Sections were re-blocked with NGS/PBS/BSA for 1 h then incubated with mouse monoclonal antibodies anti-CD31 (DAKO; 1 in 20 in block), anti-CD68 (DAKO; 1 in 20 in block) or anti-α smooth muscle actin (αSMA, DAKO; 1 in 500 in block)

overnight at 4°C. Sections were washed and incubated with the fluorochrome streptavidin 546 Alexafluor (Molecular Probes) diluted 1 in 200 in PBS for 1 h.

Sections that were labelled with anti-11βHSD1 were re-blocked with normal donkey serum (NDS)/PBS/BSA for 1 h and then incubated with sheep anti-11βHSD2 (kind gift from Prof. Ian Mason, The University of Edinburgh, UK) diluted 1 in 50 in donkey serum. Sections were washed and incubated with donkey anti-sheep peroxidase (DAKO) 1 in 200 in NDS/PBS/BSA for 30 min before washing and incubating for 10 min with tyramide Cy3 (TSA plus cyanine 3 system; Perkin-Elmer Life Sciences) as described in section 2.1.10.2.3 to amplify the 11βHSD2 immunostaining with red fluorescence.

Nucleic acids were labelled with To-Pro 3 and washed and mounted in Permafluor (Beckman Coulter). Fluorescent images were captured using an LSM 510 Axiovert 100M confocal microscope as describe in section 2.1.10.4. Images of 11βHSD1 and 11βHSD2 were analyzed comparatively by standardising the computer settings for each isoform. Therefore the relative intensity of staining for each isoform approaches the relative abundance of protein levels.

4.2.9 Measurement of net 11βHSD oxidoreductase activity

Inter-conversion of cortisone to cortisol via 11-oxoreductase activity was assessed in the presence and absence of hCG. Pooled luteinised granulosa cells were either stimulated with 100 ng/ml of hCG in serum-free medium or serum-free medium alone for 24 h. Controls included incubations containing no cells with only matrigel. After hCG stimulation, culture media was discarded from wells and replaced with culture medium containing 100 nM of cortisone substrate and 0.1 μCi of [3H] cortisone to give a final volume of 500 μl/well. All incubations were in triplicate for 4 h at 37°C with 95% air-5%CO_2. After incubation, media was added to glass tubes containing 5 ml aliquots of dichloromethane and vortexed thoroughly. To separate the aqueous and organic phases, tubes were centrifuged at 12,000 rpm for 10 min. After the aqueous phase was removed, samples were evaporated to dryness under nitrogen gas at 45°C. Steroid residues were re-suspended in 100 μl of dichloromethane and samples along with one [3H]-cortisol and one [3H]-cortisone blot were transferred to silca gel-precoated plastic sheets for thin-layer chromatographic (TLC) separation of precursor and product in the solvent system of

chloroform:ethanol (92:8 by vol) (Merck, Hoddesdon Herts UK). TLC plates were then scanned using a Bioscan System 200 detector (Lablogic Systems, Sheffield UK) and corresponding peaks were analysed for enzymatic activity in each sample and consequently each treatment group. Results are expressed as amount of cortisone converted to cortisol (pmoles) per hour.

4.2.10 Measurement of progesterone concentrations in the culture medium of luteinised granulosa cells

Progesterone concentrations were measured from collected culture medium using a plate modification of a standard progesterone RIA (Djahanbakhch *et al.*, 1981) kindly performed by Nancy Evans (HRSU MRC, Edinburgh). The intra-assay co-efficient of variation was <4% and the inter-assay co-efficient of variation was <11%. The detection limit of the progesterone assay was 0.1 nmol/litre.

4.2.11 Gelatin zymography

Gelatin zymography was performed on the culture medium from cortisol treated fibroblast-like cell cultures as described in section 2.1.11.

4.2.12 Statistical analysis

Statistical analyses used are highlighted in the figure legends. Parametric statistics were used if the data was normally distributed with similar standard deviations. Groups were analysed by ANOVA with Bonferroni pairwise comparisons; if the data was not normally distributed non-parametric statistics were used. Groups were analysed by Kruskal-Wallis test with Dunn's Multiple Comparison Test. A paired t-test was used when treatment and control samples were analysed. Differences were considered significant at $p<0.05$ level.

4.3 Results

4.3.1 Expression and localisation of 11βHSDs in the human corpus luteum

The granulosa-lutein cells of the corpus luteum can easily be identified by their localisation and morphological characteristics (Figure 4.1). Both isoforms of the cortisol metabolising enzyme 11βHSD (type 1 and type 2) could be immunolocalised to the cytoplasm of granulosa-lutein cells in human corpora lutea (Figure 4.1B,C). Specific immunostaining for each isoform could be detected in each corpus luteum analysed from each stage of the functional luteal phase. No staining could be detected in negative control sections (Figure 4.1C inset). The corpus luteum of women therefore has the capacity for the local production and metabolism of cortisol.

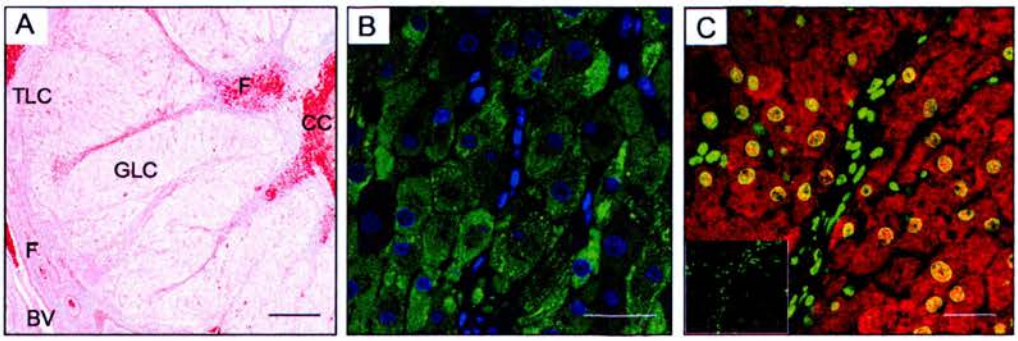


Figure 4.1 Immunolocalisation of cortisol metabolising enzymes in the human corpus luteum. **A,** A mid-luteal human corpus luteum (stained with Masson's Trichrome) is a heterogeneous tissue comprised of the steroidogenic granulosa-lutein (GLC) and theca-lutein (TLC) cells; inner and outer supportive stromal layer which consists of predominantly fibroblast (F) layers and blood vessels (BV); and the central clot (CC) that was once comprised the antral cavity of the follicle **B,** Immunofluorescence staining demonstrates that the GLC of the corpus luteum express the cortisol metabolising enzymes 11βHSD1 (green with blue nuclear stain) and **C,** 11βHSD2 (red with blue nuclear stain). Negative control shown as inset in 1C. Scale bars A=200 μm; B,C=40 μm

4.3.2 Expression and localisation of genomic glucocorticoid receptors in the human corpus luteum.

Nuclear glucocorticoid receptor immunostaining could also be detected in human corpora lutea. GR were also localised to the nuclei of granulosa-lutein cells (Figure 4.2A), but unlike the 11βHSD isoforms, GR could be localised to non-steroidogenic cell types. Dual staining immunohistochemistry with α-SMA demonstrated GR expression in stromal myofibroblasts and pericytes (Figure 4.2B,C). Dual immunostaining with CD68 and CD31 demonstrated specific nuclear GR expression in macrophages (Figure 4.2 D) and endothelial cells (Figure 4.2E) in each corpus luteum examined. This pattern of GR immunostaining was detected in corpora lutea from each stage of the luteal phase. Various different cells in the human corpus luteum therefore have the capacity to respond to both systemic and locally produced cortisol, in an autocrine, paracrine or endocrine manner.

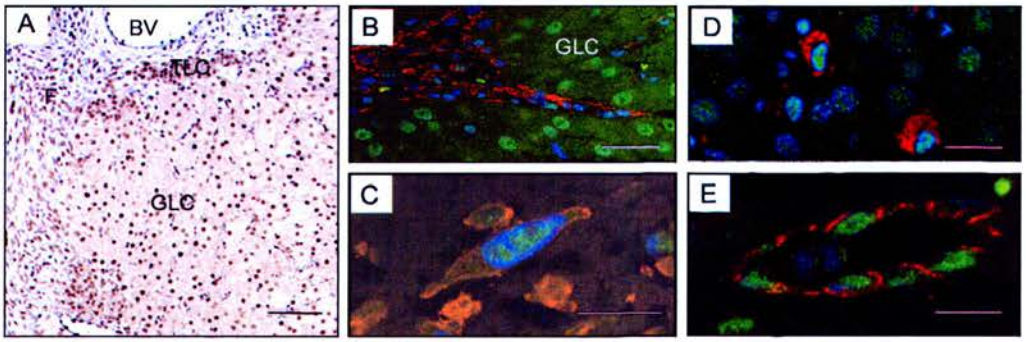


Figure 4.2 Localisation of the glucocorticoid receptor in the human corpus luteum. **A,** The GR protein is ubiquitously expressed in the corpus luteum as seen by the positive brown DAB staining with light microscopy. **B,** Immunofluorescence however further confirms that GR can be co-localised with other cells types. For example, nuclear GR (green) is present in the GLC whilst αSMA-positive cells represent stromal myofibroblasts and pericytes (red). **C,** High power images reveal that GR (green) is localised to the presumptive fibroblast cells (red) **D,** macrophages (red, CD68) and **E,** endothelial cells (red, CD31) of the corpus luteum. Nuclear staining depicted in blue. GLC= granulosa-lutein cell, TLC=thecal lutein cells, BV= blood vessel.
Scale bars E=40 μm; D=100 μm; F,H=10 μm; G=20 μm.

4.3.3 The effect of hCG on 11βHSD and GR expression in primary cultures of luteinised granulosa cells.

In order to determine whether the expression of GR and cortisol metabolising enzymes in steroidogenic cells could be acutely regulated, the effect of hCG was examined in primary cell cultures of human luteinised granulosa cells. The addition of hCG for 24 h resulted in a 30-fold up-regulation of 11βHSD1 expression ($p<0.05$, Kruskal-Wallis) (Figure 4.3A). At the same time 11βHSD2 was down-regulated ($p<0.05$, Kruskal-Wallis) (Figure 4.3B) while GR expression was also up-regulated ($p<0.05$, ANOVA) (Fig. 2C). In order to determine the functionality of the enzyme and the direction of 11βHSD activity 11-oxoreductase activity was assessed in the presence and absence of hCG. This confirmed that hCG stimulated reductase activity to promote cortisol generation ($P<0.05$, t-test) (Figure 4.3E).

As luteinised granulosa cells have genomic progesterone receptors, and hCG stimulates progesterone secretion (range 460-890 ng/ml), cells were cultured with the progesterone synthesis inhibitor aminoglutethimide to separate hCG effects from progesterone effects (Duncan *et al.*, 2005b) (Figure 4.3D). Aminoglutethimide reduced progesterone (range 160-400 ng/ml) secretion to control levels (170-200 ng/ml respectively) in the presence of hCG (Figure 4.3D) ($p<0.0001$, ANOVA). While aminoglutethimide had no effect on hCG-stimulated up-regulation of 11βHSD1 and GR it inhibited the hCG-induced down-regulation of 11βHSD2 (Figure 4.3B). This suggests that progesterone may be involved in the local regulation of 11βHSD isoform expression. Overall, these data suggest that cortisol metabolism and reception may be hormonally regulated in luteal steroidogenic cells.

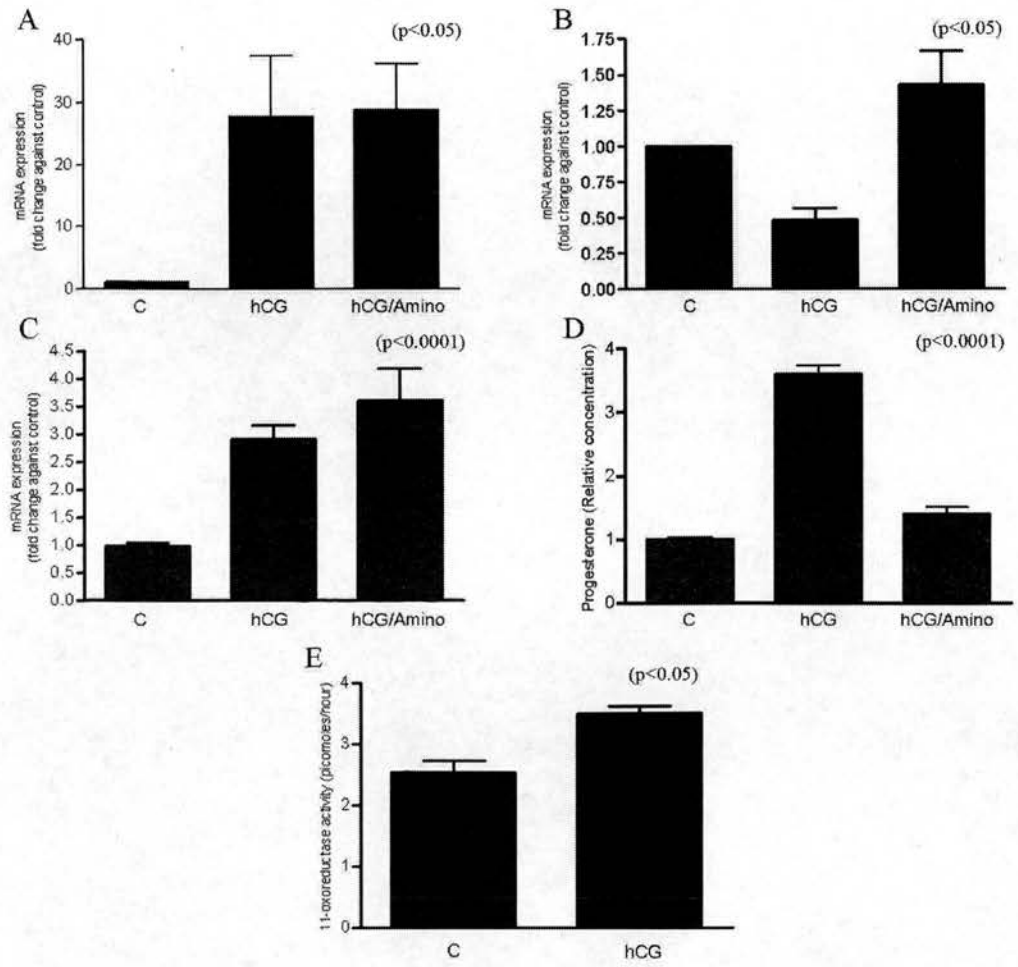


Figure 4.3 HCG modulates the 11βHSD enzymes and GR in luteinised granulosa cells in primary culture. **A,** The expression of 11βHSD1 is up regulated by hCG independently of progesterone as evident by the addition of progesterone synthesis inhibitor aminoglutethimide ($p<0.05$, Kruskal-Wallis). **B,** HCG however decreased the expression of 11βHSD2 ($p<0.05$, Kruskal-Wallis), whilst in the presence of aminoglutethimide inhibition of the type 2 isoform was prevented. **C,** Similar to that of 11βHSD1, hCG increased GR expression in luteinised granulosa cells ($p<0.0001$, ANOVA) whilst the addition of aminoglutethimide had no effect. **D,** HCG stimulated an increase of progesterone ($p<0.0001$, ANOVA) and this increase was blocked by the addition of aminoglutethimide. **E,** HCG increases 11-oxoreductase activity which acts to generate cortisol ($p<0.05$, t-test) . n.s.=no significant difference.

4.3.4 The effect of chronic manipulation of hCG in cultures of human luteinised granulosa cells.

In order to determine if hCG could regulate the expression of 11βHSDs and GR in more physiologically relevant prolonged culture conditions, 12-day cultures of luteinised granulosa cells, designed to mimic the luteal phase (Duncan *et al.*, 2005b), were examined. Withdrawal of hCG in culture down-regulated 11βHSD1 ($p<0.05$, Kruskal-Wallis) while its expression was maintained in the presence of hCG (Figure 4.4A). Conversely hCG withdrawal had no effect on 11βHSD2 expression while maintenance of hCG did not alter its expression as the trend towards reduction did not reach significance ($p>0.05$, Kruskal-Wallis) (Figure 4.4B). The expression of GR showed a similar pattern to that of 11βHSDI but there were no significant changes detected (Figure 4.4C) ($p>0.05$, ANOVA). These data suggest that cortisol metabolising enzymes may continue to be differentially regulated by hCG under chronic conditions.

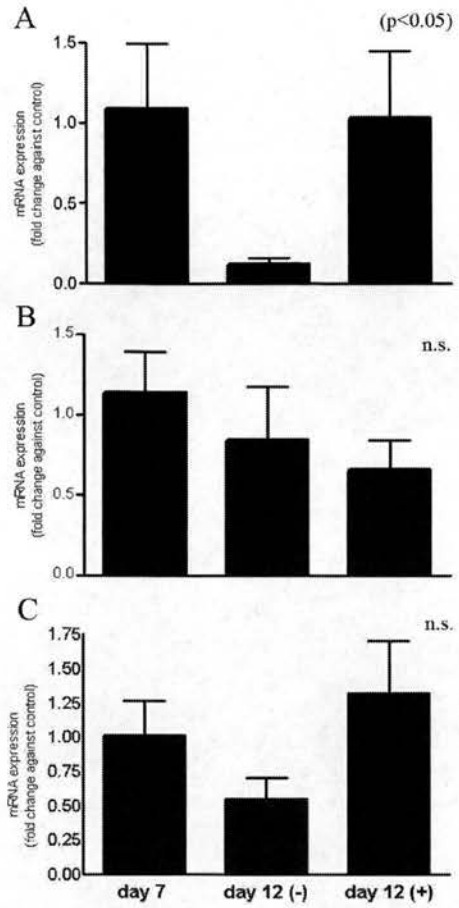


Figure 4.4 Chronic manipulation with hCG in cultures of luteinised granulosa cells designed to mimic the luteal phase involved low dose hCG stimulation until day 7, which was either replaced by maximal dose hCG on day 12 (+) or removal of hCG on day 12(-). **A,** Withdrawal of hCG at day 12 down-regulated the expression of 11βHSD1 ($p<0.05$, Kruskal-Wallis) whilst expression was maintained in the presence of hCG. **B,** Expression levels of 11βHSD2 remained unchanged ($p>0.05$, Kruskal-Wallis) by hCG withdrawal or maintenance. **C,** The expression of GR demonstrated a tendency to decrease with hCG withdrawal and maintain levels with hCG ($p<0.05$, ANOVA). n.s. = no significant difference.

4.3.5 The effect of hCG on the expression of 11βHSDs in human corpora lutea *in vivo*.

In order to determine the effect of hCG of luteal 11βHSD expression in women, archival tissues collected in the late-luteal phase in the absence or presence of exogenous hCG to rescue the corpus luteum and mimic the changes of early pregnancy were examined (Duncan *et al.*, 1998a). *In vivo*, the effects of hCG did not quite reach significance ($p>0.05$, t-test), although there was a clear differential effect on the expression of 11βHSD1 and 11βHSD2 (Figure 4.5). Exogenous hCG tended to increase 11βHSD1 (Figure 4.5A) while tending to reduce 11βHSD2 (Figure 4.5B). There were no specific effects of hCG on the expression of GR in the corpus luteum of women (Figure 4.5C). These data suggest that the differential regulation of cortisol metabolism in luteinised granulosa cells *in vitro* may also occur in the human corpus luteum *in vivo*.

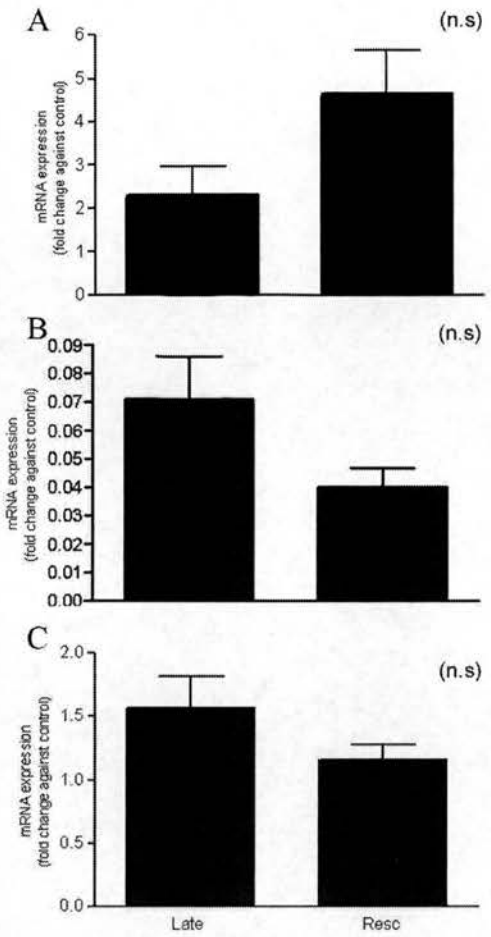


Figure 4.5 Modulation of 11βHSDs and GR in human corpora lutea *in vivo* did not reach significance during hCG rescue. However a tendency for cortisol regeneration during hCG rescue. **A,** The relative expression levels of 11βHSD1 tended towards an increase during hCG-induced luteal rescue compared with that of the late-luteal phase. **B,** Conversely the opposite was true for 11βHSD2 whereby expression levels tended towards a decline during hCG-induced luteal rescue compared to the late-luteal phase. **C,** Expression of GR remained relatively unchanged in the presence or absence of hCG. n.s. = no significant differences. (n=6 per group).

4.3.6 The effect of RU486 on primary cultures of luteinised granulosa cells

As hCG seems to promote the generation and action of cortisol it was hypothesised that cortisol may function as a local luteotrophic factor during luteal rescue and that cortisol withdrawal may have a role during luteolysis. It was therefore investigated whether cortisol could function as a survival factor in cultures of luteinised granulosa cells. The cortisol and progesterone rececptor antagonist RU486 reduced the number of luteinised granulosa cells in culture ($p<0.001$, ANOVA) after seven days treatment (Figure 4.6). This effect was not seen when progesterone synthesis was inhibited by aminoglutethimide (Figure 4.6). This decrease in cell number in the presence of RU486 could be prevented by the addition of cortisol ($p>0.05$, ANOVA) so that cell numbers were no different to controls (Figure 4.6). Conversely, the addition of saturating concentrations of progesterone or hCG in the presence of RU486 did not increase the cell number beyond that of RU486 alone ($p>0.05$, ANOVA) (Figure 4.6). These data crudely suggest that cortisol may have direct actions on luteinised granulosa cells *in vitro*.

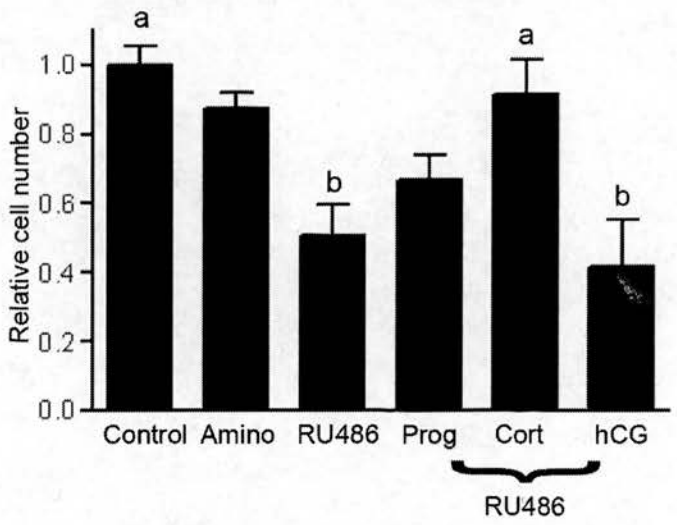


Figure 4.6 The effect of RU486 and steroids on luteinised granulosa cell survival after seven days in culture. Culturing the cells with aminoglutethimide had no effect on the number of remaining after seven days ($p>0.05$, ANOVA), while RU486 reduced cell numbers by 50% ($p<0.001$, ANOVA). The addition of progesterone to RU486 did not return cell numbers to control levels ($p<0.05$, ANOVA) whereas the addition of cortisol increased cell numbers ($p<0.05$, ANOVA) to control levels ($p>0.05$, ANOVA). The addition of hCG to RU486 had no effect ($p>0.05$, ANOVA) with numbers less than controls ($P<0.01$, ANOVA) and RU486 with cortisol ($p<0.01$, ANOVA).

4.3.7 The effect of cortisol on non-steroidogenic cells

As well as the steroidogenic cells of the corpus luteum GR can be localised to non-steroidogenic cells. This suggests that cortisol has the potential to function as a paracrine regulatory factor. Therefore, the current study investigated the effect of cortisol on novel primary cultures of ovarian fibroblast-like cells derived from the luteinising follicle (Duncan *et al.*, 2005b; Myers *et al.*, 2007a). These cells secrete MMP-2 that is regulated in a paracrine manner by hCG through intermediary molecules (Duncan, 2000). *In vivo*, hCG inhibits the expression of luteal fibroblast MMP-2 ($p<0.05$, t-test) (Figure 4.7A). *In vitro* the fibroblast-like cells express MMP-2 mRNA and proteolytic activity. The addition of exogenous cortisol inhibited both the expression ($p<0.001$, t-test) and activity ($p<0.0001$, t-test) of MMP-2 (Figure 4.7B,C). This suggests that cortisol may have the potential to have relevant effects on neighbouring non-steroidogenic cells.

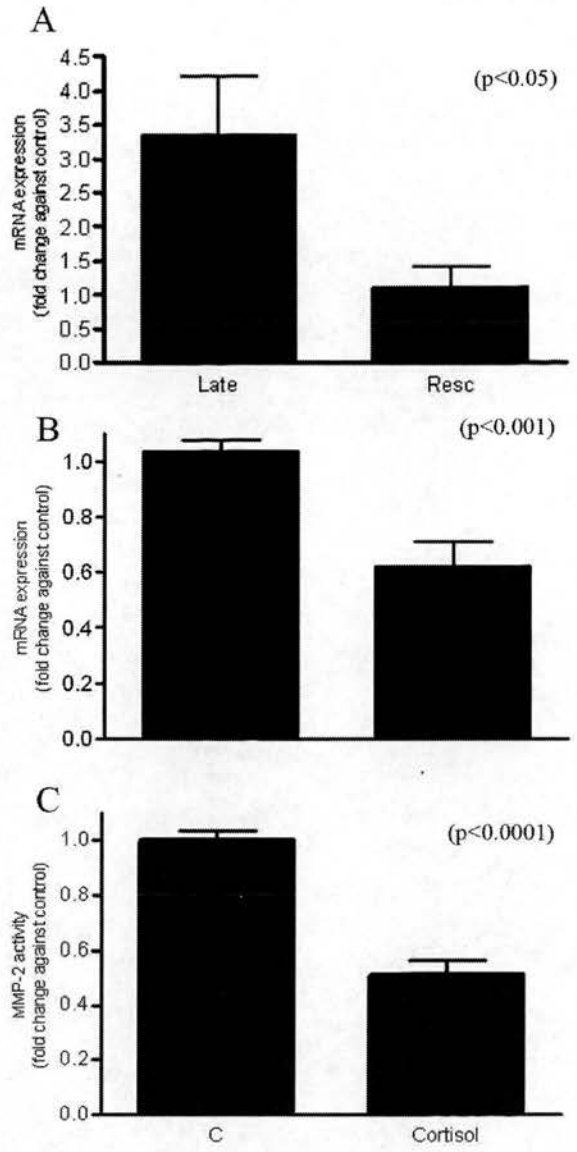


Figure 4.7 MMP-2 expression and activity appear to be modulated by glucocorticoids in primary cell cultures of fibroblast-like cells. **A,** The expression of MMP-2 was down-regulated during hCG-induced luteal rescue in human corpora lutea ($p<0.05$, t-test). **B,** Primary cell cultures of fibroblast-like cells treated with cortisol mirror the effect of hCG during luteal rescue as cortisol inhibits MMP-2 expression ($p<0.001$, t-test). **C,** Gelatin zymography further confirms that cortisol reduces MMP-2 activity ($p<0.0001$, t-test) in these fibroblast-like cells from the luteinising follicle.

4.4 Discussion

This chapter hypothesised that locally produced cortisol may be involved in regulating tissue remodelling during luteolysis. The present study has shown that the human corpus luteum expresses the enzymes required to increase or decrease the local availability of cortisol and the receptor pathways required to respond to glucocorticoids. Combination studies in human corpora lutea with primary human cell culture models to show that the expression of the 11βHSD enzymes, that control the local availability of cortisol, is hormonally regulated. As discussed previously, luteinised granulosa cells *in vitro* are not a perfect representation of granulosa-lutein cells *in vivo* (Myers *et al.*, 2007a) and for this reason the *in vitro* and *in vivo* paradigms are complementary. Both paradigms suggest that in women there is a change in 11βHSD isoforms across the luteal phase that is associated with the functional state of the gland. This is complimentary to the only other study investigating 11βHSD in the corpus luteum that suggested there may be more 11βHSD2 in the regressing rat corpus luteum (Waddell *et al.*, 1996).

It is uncertain what role cortisol has in the human corpus luteum. Roles for active glucocorticoids in the ovary have certainly been proposed and described during folliculogenesis and ovulation (Michael *et al.*, 2003). Previous experiments have demonstrated that hCG has the ability to regulate many different cell types and their molecular functions within the corpus luteum and highlighted the important role for locally produced intermediate regulatory molecules (Duncan, 2000; Duncan *et al.*, 2005b; Fraser *et al.*, 2005; Myers *et al.*, 2007a). Herein, it is hypothesised that glucocorticoids may be regulatory molecules with signalling roles within the corpus luteum. This may be a result of hCG, which acts to promotes the generation of active glucocorticoids which have a luteotrophic role in the human corpus luteum such that the structural remodelling associated with luteolysis is inhibited and maintenance of early pregnancy is facilitated. Similarly, removal of local cortisol may facilitate luteal regression in the absence of hCG.

The 11βHSDs catalyse the inter-conversion of active (cortisol) and inactive (cortisone) glucocorticoids by isoforms type 1 and 2 respectively. The temporal and spatial expressions of both isoforms have been documented in the ovary of other species (Ricketts *et al.*, 1998; Smith *et al.*, 2000; Tetsuka *et al.*, 1997; Yong *et al.*, 2000). It is well established that 11βHSD2 is the predominant isoform during the follicular phase of the ovarian cycle, localised to the non-luteinised granulosa cells prior to the LH surge

(Tetsuka *et al.*, 1997). This is an important concept as too much cortisol during the follicular phase is reported to disrupt FSH-stimulated granulosa cell development/function (and presumably oestradiol production) (Hsueh and Erickson, 1978), that would consequently inhibit successful folliculogenesis. In addition, during folliculogenesis, the predominant steroid is oestradiol, and unlike progesterone (as discussed below), oestradiol has a very low affinity for CBP (Dunn *et al.*, 1981) and thus will not displace cortisol. As it has also been reported that oestradiol can increase the hepatic production of CBP (Hammond, 2002), another mechanism may exist during the follicular phase to lower free cortisol. It seems that in the follicular phase local cortisol generation and action tends to be inhibited.

This is not the case, however, in the peri-ovulatory period. Once the dominant follicle is exposed to the mid-cycle pre-ovulatory gonadotrophin surge, the predominant isoform switches from type 2 to type 1 11βHSD (Tetsuka *et al.*, 1997; Yong *et al.*, 2000). This phenomenon can also be seen by a rise of free cortisol that is 50-times higher in follicular fluid after the LH surge (Andersen and Hornnes, 1994; Harlow *et al.*, 1997), indicative that expression levels of the 11βHSD enzymes in the ovary are an accurate measure for the direction of glucocorticoid biosynthesis. Indeed it has been suggested that this process is involved in facilitating fertility. Some studies report that IVF patients with higher cortisol to cortisone ratio in their follicular fluid have greater pregnancy success rates (Lewicka *et al.*, 2003; Michael *et al.*, 1999) although others do not agree (Andersen and Hornnes, 1994; Andersen *et al.*, 1999). It is likely however that the switch in isoforms around ovulation has effects on the oocyte as well as regulating the inflammatory reaction associated with follicular rupture and its resolution (Hillier and Tetsuka, 1998). Such findings further support the hypothesis that 11βHSD enzyme activities in the human ovary are developmentally and hormonally regulated (Yong *et al.*, 2000).

The expression of 11βHSD1 in luteinising granulosa cells is maintained in the granulosa lutein cells of the corpus luteum. Additionally, in the present study 11βHSD2 was also detected in the corpus luteum. Type 2 11βHSD mRNA expression and protein has previously been reported to be very low or undetectable in freshly isolated luteinised granulosa cells of both rats and humans (Michael *et al.*, 1997; Tetsuka *et al.*, 1999; Tetsuka *et al.*, 1997; Thurston *et al.*, 2003). This suggests that as the corpus luteum is formed there may be a recovery of 11βHSD2 expression. Indeed all cultures of luteinised

granulosa cells expressed 11βHSD2 and they had been cultured for at least seven days before analysis. Although 11βHSD1 remains the most abundant isoform in luteinised granulosa cells in culture and in the corpus luteum both isoforms are expressed. HCG inhibited the expression of 11βHSD2 *in vitro* and tended to do the same *in vivo*. In contrast, hCG stimulated 11βHSD1 expression acutely, and in prolonged cultures, and tended to do the same *in vivo*. The regulation of 11βHSD1, like in the peri-ovulatory period, seems to a direct effect of hCG signalling. However as the inhibition of 11βHSD2 by hCG was blocked when progesterone synthesis was inhibited, progesterone rather than hCG may be involved in the inhibition of 11βHSD2 expression. This finding supports a similar experiment in granulosa cells by Thurston et al. (Thurston *et al.*, 2003) and demonstrates the same principles of progesterone actions observed in the kidney and placenta (Lopez Bernal *et al.*, 1980; Quinkler *et al.*, 1999; Sun *et al.*, 1998) whereby progesterone inhibits 11βHSD2. Indeed, as progesterone receptor expression is down-regulated as the corpus luteum matures this may be a mechanism for the re-emergence of steroidogenic cell 11βHSD2 expression in the corpus luteum (Duncan *et al.*, 2005a).

The effect of hCG on 11βHSD1 expression was mirrored by 11-oxoreductase activity levels and the generation of cortisol from cortisone. Unfortunately, it is not possible to assess cortisol metabolism in the late-luteal and rescued corpus luteum to confirm this *in vivo*. However it is believed that changes in the expression of different 11βHSD isoforms in tissues informs the direction of cortisol/cortisone metabolism (Yong *et al.*, 2000) and it is clear that 11βHSD1 has the potential to act as a bi-directional enzyme (given the appropriate co-enzyme environment) (Morris *et al.*, 2003). However, previous detailed studies on the direction of metabolism in luteinised granulosa cells (Thurston *et al.*, 2003; Yong *et al.*, 2000), ovarian surface epithelial cells (Rae *et al.*, 2004a; Rae *et al.*, 2004b; Yong *et al.*, 2002) and other tissues (Jamieson *et al.*, 2000; Seckl and Walker, 2004; Walker and Seckl, 2003) as well as the phenotype of 11βHSD knockout mice (Hadoke *et al.*, 2001) have suggested that *in vivo* 11βHSD1 preferentially generates cortisol. However as pointed out by Jonas et al., recent studies have established that the net direction of 11βHSD1 is dependant upon the cells availability of hexose-6-phosphase dependent NADPH that may be different in high steroidogenically active tissues (Jonas *et al.*, 2006) and the direction of cortisol metabolism *in vivo* remains to be studied.

The human corpus luteum has the potential to respond to locally generated cortisol as it expresses nuclear GR. Indeed GR has been localised previously to many cell compartments in the ovary (Tetsuka *et al.*, 1999). Although the primary receptor for cortisol is GR, cortisol also has a high affinity for the mineralocorticoid receptor (MR). The expression of MR was not analysed in the present study however it is reported to be expressed in the ovary (Tetsuka *et al.*, 1999). The GR is localised to the nuclei of steroidogenic cells and these cells of the human corpus luteum are also reported to express other important nuclear steroid receptors such as oestrogen and progesterone receptors (Hosokawa *et al.*, 2001; Maybin and Duncan, 2004; Misao *et al.*, 1999). It is unclear whether other steroids can influence glucocorticoid signalling by receptor dependent mechanisms but it is likely that cortisol has direct effects on the cells expressing the 11βHSD enzymes involved in its synthesis and metabolism.

It is not clear if the expression of GR in the corpus luteum is regulated. Duncan et al., have previously shown that steroidogenic cell progesterone receptors are differentially regulated in the corpus luteum across the luteal phase (Duncan *et al.*, 2005a) although their role has not yet been elucidated (Stouffer, 2003). In contrast, there were no obvious changes in steroidogenic cell GR immunostaining across the luteal phase. Indeed in the endometrium, where glandular progesterone receptor expression in the secretory phase shows changes similar to that in luteal steroidogenic cells (Duncan *et al.*, 2005a; Koh *et al.*, 1995), there was no change in GR expression across the functional menstrual cycle (McDonald *et al.*, 2006). However, *in vitro* hCG, tended to up regulate GR expression similar to its effects on 11βHSD1. Whether GR expression in corpora lutea is hormonally regulated but masked by detection methods is not entirely clear. What is clear is that multiple cell types in each corpus luteum express nuclear GR.

Luteal endothelial cells and macrophages express nuclear GR. Protein co-localisation of GR with CD31 demonstrates specific nuclear staining of endothelial cells. The effect of cortisol on luteal endothelial cells is not clear but glucocorticoids have been shown to suppress angiogenesis (Small *et al.*, 2005) and this is most notably due to the suppression of vascular endothelial growth factor (VEGF) action (Nauck *et al.*, 1998; Small *et al.*, 2005). In the human corpus luteum, however, there continues to be some angiogenesis stimulated by hCG during luteal rescue (Wulff *et al.*, 2001) in response to the marked up-regulation of VEGF after exogenous hCG (Wulff *et al.*, 2000). Immune cells, most

notably macrophages (CD68+ve cells), that also express GR, accumulate in the corpus luteum during luteolysis and show a marked reduction in number during hCG-induced luteal rescue (Duncan *et al.*, 1998b). The role of cortisol in luteal macrophage accumulation and action is not clear but as glucocorticoids are known to regulate cytokine signalling and macrophages in both health and disease (Rogatsky and Ivashkiv, 2006) it may affect the immune cell mediated processes during luteolysis. Luteal myofibroblasts are known to express macrophage chemotractant protein-1 (MCP-1) (Senturk *et al.*, 1999) and these cells also express nuclear GR.

When considering a role for glucocorticoids in the corpus luteum it is very important to establish the relationship between cortisol and the marked excess of, structurally related, progesterone. Cortisol exists in one of two forms, bound and free, that regulate its bioavailability. In most systems, the majority of the steroid is bound to plasma proteins (notably CBP) with only a fraction free and unbound (Andersen, 2002). Although CBP has the highest affinity for cortisol of all the binding globulins, other steroids such as progesterone and particularly 17OH-progesterone have high binding affinities to CBP (Andersen, 2002; Dunn *et al.*, 1981). Therefore very high concentrations of progesterone and progesterone metabolites (such as in the corpus luteum) will displace cortisol from CBP, that acts as a buffer reservoir, resulting in the environment becoming enriched with free cortisol. This scenario is known as the 'free hormone hypothesis' (Andersen, 2002; Dunn *et al.*, 1981) and predicts that the bioactivity of cortisol is proportional to free hormone concentrations and not total concentration which includes the protein-bound fraction. This is an important paradigm to consider as the concentration of free biologically active cortisol in pre-ovulatory follicular fluid is 10 times greater than that of serum (Andersen and Hornnes, 1994). It is likely that, because of high local progesterone concentrations, rather than being mainly bound to CBP in the corpus luteum, locally generated cortisol is more likely to be free and functional at lower concentrations.

It is still not clear what effects cortisol has on luteal cells. This chapter hypothesised that it may affect the survival of luteinised granulosa cells. The current observations and those of others (Rothchild, 1996) suggested that RU486 reduced survival of these cells in culture. Utilising simple cell counting to document the effect on RU486 on cell survival and treating cells with the progesterone synthesis disruptor aminoglutethimide, that blocks $P450_{scc}$, the current study showed no change in their morphology or viability.

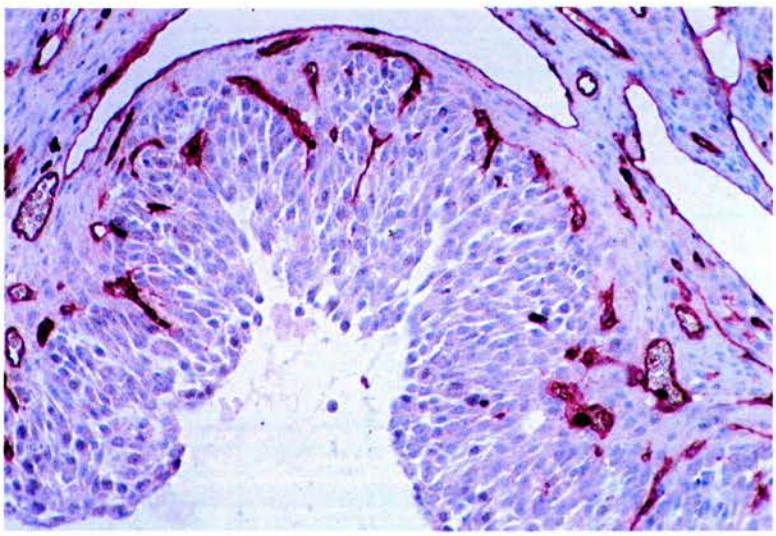
Furthermore, the effect of RU486 could not be fully reversed by hCG or exogenous progesterone. Unlike a previous study using human luteinised granulosa cells, under slightly different conditions (Rothchild, 1996), the present study could not fully reverse the RU486 effects using progesterone. Surprisingly however, the effect of RU486 was reversed using cortisol. It is not clear if this effect is at the level of the receptor as the concentration of cortisol would not be expected to fully displace the RU486. It may be that cortisol affects the cell susceptibility to RU486 in different ways. There may be specific effects of RU486 not mediated by hormone antagonism. Direct actions of RU486 have been reported in ovarian epithelial cancer cells and human endometrium, whereby it down-regulated molecules involved in signal transduction pathways by cytokines, growth factors and other physiological stimuli that control cell functions (Catalano *et al.*, 2003). Indeed in cultured luteinised granulosa cells, cortisol and dexamethasone offer protection against serum deprivation and induced apoptosis (Sasson and Amsterdam, 2003; Sasson *et al.*, 2001) through mechanisms that include stabilisation of the actin cytoskeleton (Sasson and Amsterdam, 2002). Whatever the mechanism of action, these results crudely suggest that cortisol may have direct effects on luteal steroidogenic cells. Indeed if cortisol has any direct genomic effects *in vivo* they are more likely to promote, rather than inhibit, steroidogenic cell survival and function.

If cortisol does have luteotrophic actions it may also have specific actions on the non-steroidogenic cells of the corpus luteum that are key regulators of tissue remodelling during luteolysis. Luteal fibroblasts are the main source of MMP-2, a key proteolytic enzyme involved in tissue remodelling associated with luteolysis in women (Duncan *et al.*, 1998a) and in many other species (Endo *et al.*, 1993; Pitzel *et al.*, 2000; Young *et al.*, 2002). Both primates (Young *et al.*, 2002) and women (Duncan *et al.*, 1998a) show maximal MMP-2 expression in the late-luteal phase. However, during maternal recognition of pregnancy, MMP-2 production is considerably reduced (Duncan *et al.*, 1998a), suggestive that hCG is regulating the enzymatic expression through intermediate molecules (Duncan, 2000). Furthermore, it is tempting to speculate that active glucocorticoids may also prevent luteolysis by inhibition of intraluteal prostaglandin synthesis (Wang *et al.*, 1993). Whilst this is an attractive suggestion, the actual role of prostaglandins in the human corpus luteum remains elusive.

In the present study, these novel findings suggest that cortisol may be involved in paracrine interactions that control tissue remodelling. Recently, a published *in vitro* study from the current thesis demonstrated by modelling the human corpus luteum that activin A is a paracrine factor secreted from luteinised granulosa cells which may up-regulate fibroblast MMP-2 secretion (Chapter 3;(Myers *et al.*, 2007a)) in the absence of hCG. In contrast to activin A (Myers *et al.*, 2007a), this chapter demonstrates that cortisol treatment of luteal fibroblast-like cells in culture results in a reduction in the production of MMP-2, a pattern reflecting MMP-2 expression in exogenous hCG rescued luteal tissue (Duncan *et al.*, 1998a). Indeed glucocorticoids decreased MMP-2 activity in rat aortic smooth muscle cells (Pross *et al.*, 2002) and in a fibrosarcoma cell line (Saadat *et al.*, 2005). It seems that the non-steroidogenic cells forming the corpus luteum have the ability to directly respond to cortisol. If these effects do occur *in vivo* it is likely that cortisol tends to inhibit rather than stimulate the remodelling associated with luteolysis, and may therefore be considered to be luteotrophic in nature.

In the present study it was hypothesised that during luteolysis more cortisol is generated in the local environment, consequently preventing aberrant scarring to the tissue. It is clear however from the result obtained from this study that luteolysis is not associated with an increase in cortisol, and the opposite is true. This study has shown that hCG tends to generate cortisol by up-regulating 11βHSD1 and down-regulating 11βHSD2. This chapter clearly demonstrates that the corpus luteum has the potential to react to this cortisol and that the effect on steroidogenic and neighbouring cells tends to be luteotrophic rather than luteolytic. In summary, observational and interventional *in vivo* and *in vitro* models have generated results that suggest that cortisol tends to be withdrawn during luteolysis and maintained during luteal rescue. Glucocorticoids may have a role in the local luteal regulation of maternal recognition of pregnancy in women.

5 Activin A reduces luteinisation of human luteinised granulosa cells and has opposing effects to hCG *in vitro*



So far, the previous result chapters have begun to elicit the differential effects of activin A and hCG in the human corpus luteum. Chapter 3 has clearly shown that activin A may be involved in promoting luteolysis at the end stage of the luteal phase, whilst Chapter 4 has shown that hCG-derived cortisol may play a luteotrophic role during the maternal recognition of pregnancy. The focus of the current chapter was to determine if activin A was an anti-luteal agent at the start of the luteal phase and if so, could it inhibit the process of luteinisation?

(The folliculo-luteal transition in a marmoset monkey ovary, kindly provided by Prof Hamish Fraser)

5.1 Introduction

The transition of a dominant follicle into the corpus luteum is one of the fundamental processes in reproductive biology. It involves the transformation of follicular cells into the most active steroidogenic endocrine gland in the body. Luteinisation, the process whereby follicular granulosa cells differentiate into the granulosa-lutein cells of the corpus luteum involves marked and disparate morphological, biochemical and cellular changes. The granulosa cells become terminally differentiated, such that they do not divide again, and develop the enzyme machinery necessary for massive progesterone synthesis. This highly organised process, that is tightly orchestrated by steroid hormones, gonadotrophins and growth factors, is associated with structural and functional changes that characterise the folliculo-luteal transition. In a conception cycle, a viable corpus luteum is essential to the success of early gestation whilst in a non-conception cycle its demise is necessary for the next wave of folliculogenesis.

The molecular pathways involved in the luteinisation of granulosa cells are not entirely clear. Whilst the pre-ovulatory LH surge, or addition of LH or hCG during an assisted conception programme, can initiate luteinisation, experimental work clearly shows that removal of granulosa cells from the follicle causes spontaneous luteinisation in the absence of LH. Indeed this hampers the study of follicular granulosa cells *in vitro*. It is therefore believed that luteinisation is a differentiation pathway that is programmed before antral formation, and the only way the follicles can escape this fate is by inhibitory factors (Wehrenberg and Rune, 2000). Literature suggests that the follicle itself can provide a mileu in order to discourage luteinisation (Channing *et al.*, 1980; Eppig *et al.*, 1997; Murphy, 2000) and inhibitor(s) of such magnitude may actually be present in follicular fluid (Channing *et al.*, 1978; Ledwitz-Rigby *et al.*, 1977) or come directly from the oocyte itself (Brankin *et al.*, 2003; Vanderhyden and Macdonald, 1998). It therefore appears that the LH surge is able to remove such inhibitory factors, disrupt connections between granulosa cells and the oocyte and/or induce genes that facilitate luteinisation.

One molecule that may have a role in the prevention of luteinisation is activin A, a dimeric glycoprotein and member of the transforming growth factor TGF-β superfamily. Activin A is found in follicular fluid and can delay granulosa cell luteinisation and/or atresia by decreasing basal and hCG-induced progesterone secretion in human (Di Simone *et al.*, 1994; Rabinovici *et al.*, 1990), monkey (Brannian *et al.*, 1992) sheep

(Shidaifat *et al.*, 2001) and goat (Shidaifat, 2001) granulosa cells. In addition this thesis has shown that activin A action may be involved in promoting luteolysis at the end stage of the luteal phase (Chapter 3; (Myers *et al.*, 2007a)). This study shows that during maternal recognition of pregnancy one of the roles of hCG may be to inhibit activin action. Therefore it was hypothesised that activin A was anti-luteal and at the start of the luteal phase one of the roles of the preovulatory LH surge is to remove activin A and facilitate luteinisation. At the end of the luteal phase activin A action increases to promote luteolysis and in early pregnancy hCG, acting through the LH receptor, continues to inhibit activin A action and facilitate luteal maintenance.

5.1.1 Aims

To determine if activin A and hCG have opposing effects in luteinised granulosa cells *in vitro*

To establish if the luteinisation of granulosa cells involves a differentiation step that is not terminal and that activin A can return the luteinised granulosa cell to a more follicular phenotype

To determine the effects of hCG and activin A on luteal steroidogenesis

To localise the protein expression of genes which are modulated by the preovulatory LH surge in an antral follicle and corpora lutea

To establish if hCG and activin can modulate the expression of genes known to change during the follicular-luteal transition

To determine if there is a dose effect of activin A required to attenuate maximal stimulating doses of hCG in cultures of luteinised granulosa cells

5.2 Materials and Methods

5.2.1 Collection of human ovarian tissue

Normal ovarian tissue was collected (n=3) from women with regular cycles undergoing hysterectomy for benign conditions. Tissue collection was approved by the local medical research ethics committee and all women gave informed consent.

5.2.2 Isolation of human luteinised granulosa cells

Isolation of luteinised granulosa cells using Percoll gradient centrifugation was carried out as described in section 2.1.3. Twenty-four well cell culture plates were precoated with 25 µl of matrigel (BD Biosciences) and 100,000 viable cells per well were plated in 1 ml of serum-free media.

5.2.3 Activin A and hCG treatments in primary cultures of luteinised granulosa cells

Pooled luteinised granulosa cells in serum-free medium were refreshed with serum-free culture medium every 2 days after until day five when they were treated with the combinations of human recombinant activin A (R&D Systems) or a maximally stimulating dose of hCG (Serono). The following six treatments were added to luteinised granulosa cells; 1) 0.1% BSA in sterile PBS; 0.1% BSA with 2) 100 ng/ml hCG; 3) 25 ng/ml activin A; 4) 100 ng/ml activin A; 5) 25 ng/ml activin A with 100 ng/ml hCG; and 6) 100 ng/ml activin A with 100 ng/ml hCG. Each pooled experiment for the following treatments was carried out at least three times to avoid biological bias.

5.2.4 Preparation of cDNA from luteinised granulosa cells cultures

Luteinised granulosa cell mRNA was extracted using RNeasy mini-spin columns after lysis by the addition of RNeasy lysis buffer (Qiagen). Lysates were frozen until processed as per manufacturers' protocols then DNase treated with on-column DNaseI (Qiagen) and quantified using the NanoDrop ND-1000 Spectrophometer (NanoDrop Technologies).

Messenger RNA was then reverse transcribed into cDNA using random hexamers (Applied Biosystems).

5.2.5 Quantitative analysis of gene expression by RT-PCR

Quantitative real-time PCR was carried out on the ABI PRISM 7900 heat-cycler sequence detection system (Applied Biosystems) using specific primers and probes (Eurogentec) for each gene of interest (Table 5.1) and levels were related to a ribosomal 18S internal control (Applied Biosystems). All samples were performed in duplicate and a relative comparison was made to an appropriate tissue control tissue cDNA.

Table 5.1 List of all primer/probe sequences used for Taqman quantitative RT-PCR and reference obtained from.

Gene	Fwd primer 5'-3'	Rev primer 5'-3'	Probe 5'-FAM-TAMRA-3'
11βHSD1	AAGATGTTCCTGCATGGATTTC	AGCTCTGCGCCAAGAAGAAGT	TGACAGCTCACTCTGGACCACTCTTCTGA (Rae *et al.*, 2004a)
11βHSD2	GGCCAAGGTTTCCCAGTGA	GTTGTGCCAGGAGGGTGTTT	CTCTGCGCCTCTCCACTGTTTCATGA (Rae *et al.*, 2004a)
ERα NM_000125	TGATTGGTCTCGTCTGGCG	CATGCCCTCTACACATTTTCCC	TGCTCCTAACTTGCTCTTGGACAGGAACC (Henderson *et al.*, 2003)
Inhibin α subunit M13144	CTCGGATGGAGGTTACTCTTTCAA	GAAGACCCCCCACCCTTAGA	TATGAGACAGTGCCCAACCTTCTCACGC (Casagrandi *et al.*, 2003)
β-glycan NM_003243	AGCAGGTGAGGAACCCCAG	AGTGTTGTATAGCTCCATGTTGAAGG	CCAGGAACAGCCCCACGGAAACA (Casagrandi *et al.*, 2003)
Follistatin BC004107	CAGTAAGTCGGATGAGCCTGTCT	CAGCTTCCTTCATGGCACACT	TGCCAGTGACAATGCCACTTATGCCA (Casagrandi *et al.*, 2003)
InhibinβA subunit M13436	GGACATCGGCTGGAATGACT	GGCACTCACCCTCGCAGTAG	ATCATTGCTCCCTCTGGCTATCATGCC (Casagrandi *et al.*, 2003)
FSHR NM_000145	AACACCCATCCAAGGAATGG	GGGCTAAATGACTTAGAGGGACAA	TCTTCAGCTCCCAGAGTCACCAGTGGTTC (Salas *et al.*, 2006)
LHR M63108	CTGAAATACTGATCCAGAACACCAA	GCTCAAGTATTTTAATCCGGGAAGA	ATCTGAGATACATTGAGCCCGGAGCAT (Ji *et al.*, 2002)
StAR NM_000349	TTGCTTTATGGGCTCAAGAATG	GGAGACCCTCTGAGATTCTGCTT	CATGCGCTGGCAGTACATGTGCAC (Oskarsson *et al.*, 2006)
3βHSD	Assay on demand (Applied Biosystems Hs00605123_m1)		

5.2.6 Immunohistochemistry

Immunolocalisation of ERα was carried out using a mouse monoclonal antibody (Vector) in 5μm paraffin tissue sections of human ovary prepared on poly-L-lysine-coated microscope slides. These sections were dewaxed, rehydrated, washed in PBS and subjected to antigen retrieval as described in section 2.1.9. All sections were washed and blocked in 3% H_2O_2/methanol, avidin/biotin block and normal goat serum as described in sections 2.1.9. Sections were incubated overnight in primary antibody diluted 1 in 20 in NGS blocking solution at 4°C.

All sections were then washed twice for 5 min in PBS before incubation with biotinylated goat anti-mouse secondary antibody (DAKO) 1 in 500 in PBS. Incubations lasted for 1 h and were followed by two washes for 5 min. Thereafter, sections were incubated in avidin-biotin complex-HRP (DAKO) for 1 h as manufacturers' instructions. Incubations were at room temperature for 1 h and all sections were washed in PBS (2 x 5 min) and bound antibodies visualised by incubation with liquid DAB (DAKO). Sections were counterstained lightly with hematoxylin to enable cell identification. Negative controls for each antibody examined were performed identically to the above protocol but with primary antibody incubations substituted with blocking serum.

5.2.7 Fluorescent immunohistochemistry

Co-localisation studies of type 11βHSD1 and 11βHSD2 were performed as described in section 4.2.8.

5.2.8 Statistical analysis

Statistical analyses used are highlighted in the Figure legends. Parametric statistics were used if the data was normally distributed with similar standard deviations. Groups were analysed by ANOVA with Bonferroni pairwise comparisons; if the data was not normally distributed non-parametric statistics were used. Groups were analysed by Kruskal-Wallis test with Dunn's Multiple Comparison Test. A paired t-test was used when treatment and control samples were analysed. Differences were considered significant at $p<0.05$ level.

5.3 Results

5.3.1 The effects of activin and hCG on luteinised granulosa cell steroidogenic pathway

Luteinised granulosa cells express the enzymatic machinery to synthesise progesterone. Maximally stimulating doses of hCG (100 ng/ml) maintained the high LH receptor and low FSH receptor expression and increased the expression of StAR ($p<0.001$) and 3βHSD ($p<0.001$). In contrast, physiological concentrations of activin A (25 ng/ml) inhibited LH receptor expression ($p<0.01$) and induced FSH receptor expression ($p<0.05$) (Figure 5.1) In concurrence with previous studies (Brannian *et al.*, 1992; Di Simone *et al.*, 1994) activin A also negated gonadotrophin-induced progesterone secretion from luteinised granulosa cells (data not shown).

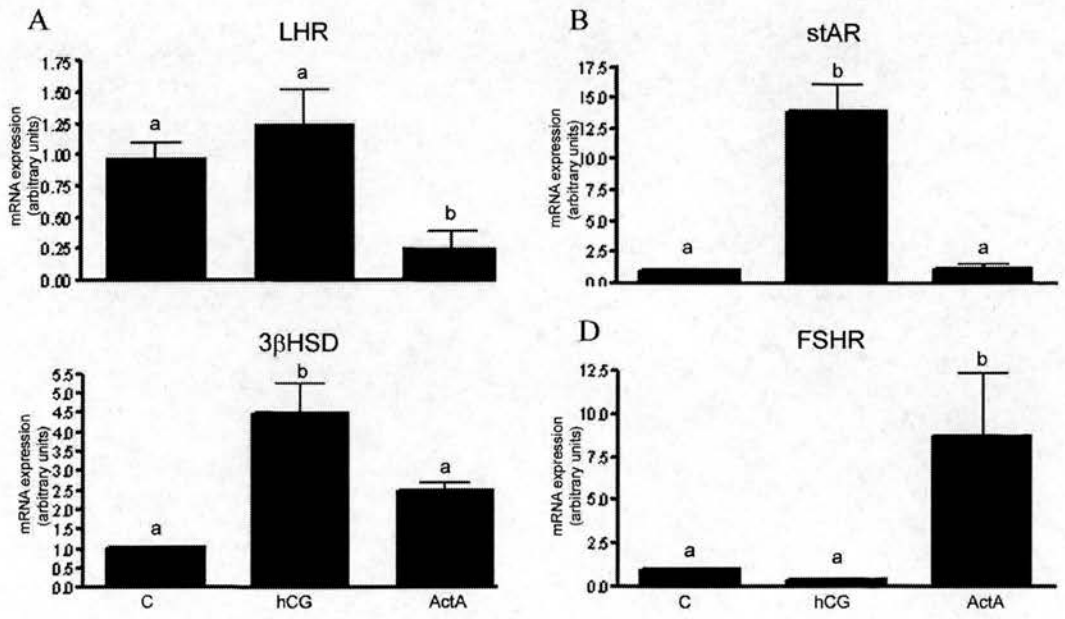


Figure 5.1 Differential effects of activin A and hCG on key genes involved in the steroidogenic pathway. **A,** HCG maintained the expression of LHR ($p>0.05$, Kruskal-Wallis) however activin A inhibited receptor expression in primary cultures of luteinised granulosa cells ($p<0.01$, Kruskal-Wallis). **B,** Key regulators of the steroidogenic pathway, StAR ($p<0.001$, Kruskal-Wallis) and **C,** 3βHSD ($p<0.001$, ANOVA) were up-regulated by hCG treatment whilst activin A had no effect upon these genes ($p>0.05$). **D,** Like LHR expression, hCG maintained the expression of FSHR *in vitro* however treatment with activin A significantly up-regulated receptor expression ($p<0.05$, ANOVA).

5.3.2 The effects of activin and hCG on the protein expression of other molecules that change during luteinisation

The 11βHSD enzyme isoforms change during luteinisation (Michael *et al.*, 1997; Tetsuka *et al.*, 1997; Thurston *et al.*, 2003; Yong *et al.*, 2000). The predominant isoform in follicular granulosa cells is 11βHSD2 (Figure 5.2 A-C) while the predominant isoform in granulosa-lutein cells of the corpus luteum is 11βHSD1 (Figure 5.2 D-F). This suggests that during luteinisation 11βHSD1 is up-regulated while 11βHSD2 is down-regulated. In addition immunolocalisation of ERα is different in the follicle and in the corpus luteum (Saunders *et al.*, 2000). Granulosa cells of the antral follicle clearly express ERα while expression in the granulosa-lutein cells of the corpus luteum appears negligible (Figure 5.2 G-H). This suggests that ERα expression is down-regulated during luteinisation.

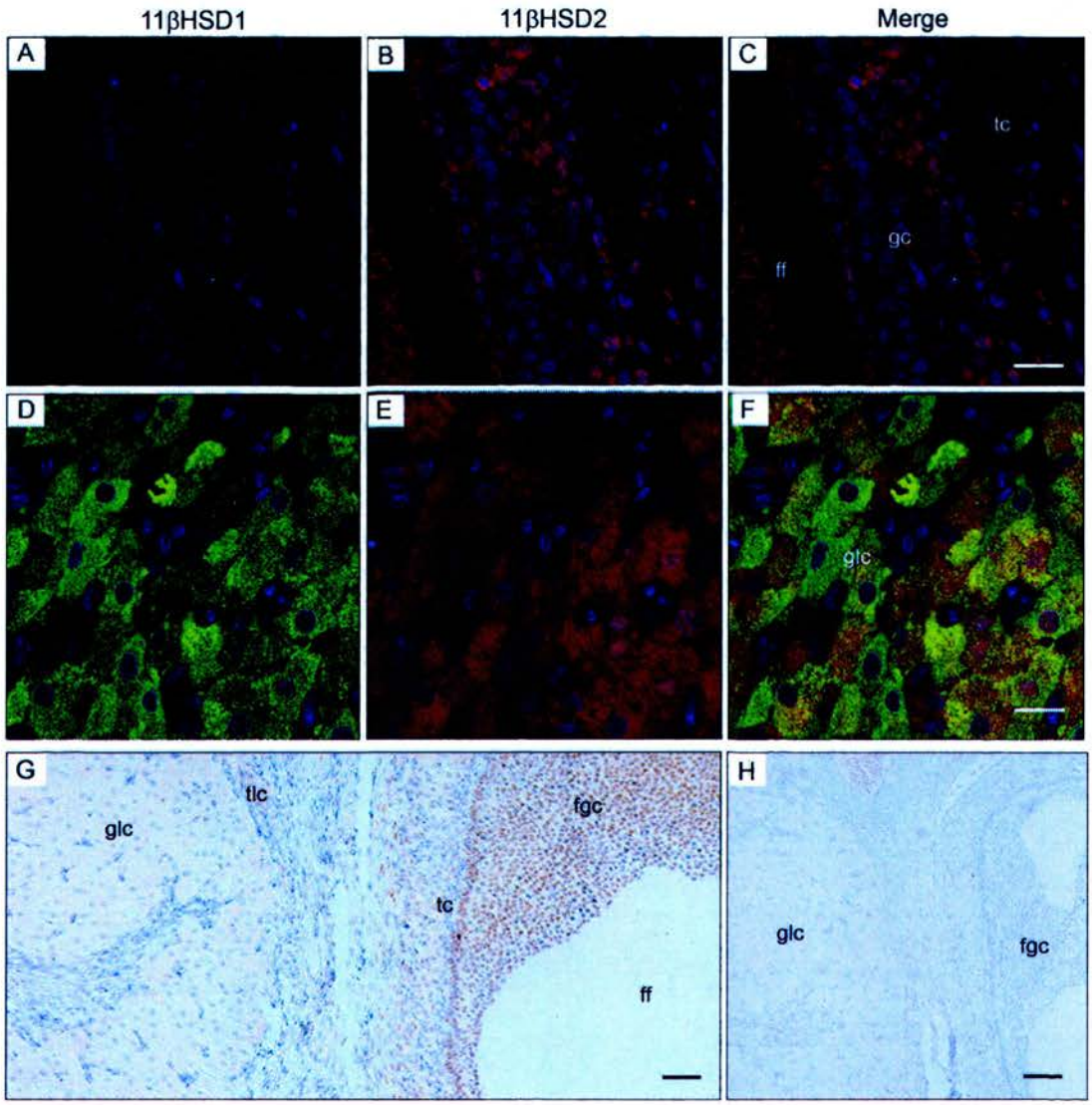


Figure 5.2 Immunolocalisation of 11βHSD types 1 and 2 and ERα in the human ovary. Double immunofluorescence of 11βHSD1 in green (panels A, D) and 11βHSD2 in red (panels B, E) in the mural granulosa cells (gc) of an antral follicle (A-C) and the granulosa-lutein cells (glc) of the corpus luteum (D-F). **A,** Single immunofluorescence clearly demonstrates that 11βHSD1 is not expressed in the granulosa cells of an antral follicle whilst, **B,** 11βHSD2 is indeed localised to these cells **C,** A merge image of panels A and B clearly showing only 11βHSD2 protein expression in the follicular granulosa cells (fgc). **D,** The steroidogenic cells of the corpus luteum express both 11βHSD1 (green) and **E,** 11βHSD2 (red). **F,** A merge image of panels D and E demonstrates predominance of 11βHSD1 isoform over 11βHSD2 in the granulosa-lutein cells (glc). **G,** Light field image of an ovarian section showing both a corpus luteum and an antral follicle demonstrates that ERα is localised to the granulosa cells of the follicle (fgc) but not the adjacent thecal cell (tc) layer. The follicular fluid in the antral cavity of the follicle is denoted by (ff). ERα staining in the granulosa-lutein cells (glc) and theca-lutein cells (tlc) of the corpus luteum is negligible. **H,** A negative control for ERα shows no staining in either cell type. Scale bars A-F=20 μm, G=40 μm, H=60 μm

5.3.3 The effects of activin and hCG on the mRNA expression of other molecules that change during luteinisation

As expected (Myers *et al.*, 2007b), hCG markedly up-regulated the expression, of 11βHSD1 ($p<0.001$), in cultures of luteinised granulosa cells by over 30-fold (Figure 5.3A). Although activin A (at either at either 25 or 100 ng/ml) alone had no effect upon 11βHSD1 expression, it was evident that when added simultaneously with hCG, activin opposed the effect of hCG ($p<0.01$) (Figure 5.3A). The opposite effect tended to be seen when 11βHSD2 isoform was examined. HCG tended to reduce, while activin A tended to increase, 11βHSD2 expression (Figure 5.3B). The action of activin A on the expression of 11βHSD2 was inhibited by hCG (Figure 5.3B). A similar pattern was seen when expression of ERα was investigated (Figure 5.3C). In cultures of luteinised granulosa cells hCG decreased receptor expression ($p<0.05$) (Figure 5.3C). Activin A (25 or 100 ng/ml) maintained ERα expression although in simultaneous treatments with hCG, hCG tended to attenuate this response (Figure 5.3C).

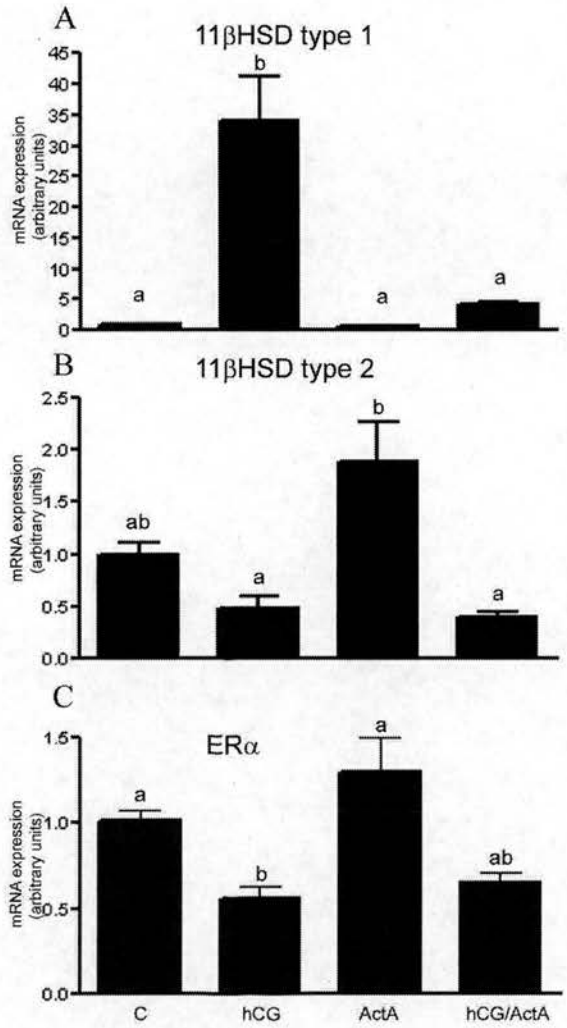


Figure 5.3 Expression of genes that change during luteinisation. **A,** HCG up-regulated 11βHSD1 by over 30-fold ($p<0.001$, ANOVA) in primary cultures of luteinised granulosa cells, whilst activin A alone had no effect ($p>0.05$, ANOVA). However when added simultaneously with hCG, activin A attenuated the hCG response ($p<0.01$, ANOVA). **B,** In contrast, 11βHSD2 tended to be up-regulated by activin A treatment, whilst hCG significantly attenuated this effect ($p<0.05$, Kruskal-Wallis). **C,** Expression of ERα was decreased by hCG *in vitro* ($p<0.05$, Kruskal-Wallis) whilst activin A maintained receptor expression. HCG added simultaneously with activin A tended to reduced expression levels of ERα similar to those of hCG.

5.3.4 Activin A inhibits hCG effects in a dose-dependent manner

To further elucidate the disparate effects of activin A and hCG, activin A was added at two different concentrations (25 and 100 ng/ml) simultaneously with hCG (100 ng/ml) to cultures of luteinised granulosa cells (Figure 5.4). Activin A and hCG had opposing effects on the expression of candidate genes in a dose-dependent fashion. Increasing concentrations of activin A significantly ($p<0.01$) decreased the expression of the LH receptor (Figure 5.4) in a dose-dependent manner in the presence of hCG and similar patterns were also observed for StAR ($p<0.001$) and 11βHSD1 ($p<0.01$) (Figure 5.4). In contrast, FSH receptor expression tended to increase with the dose of activin A (Figure 5.4).

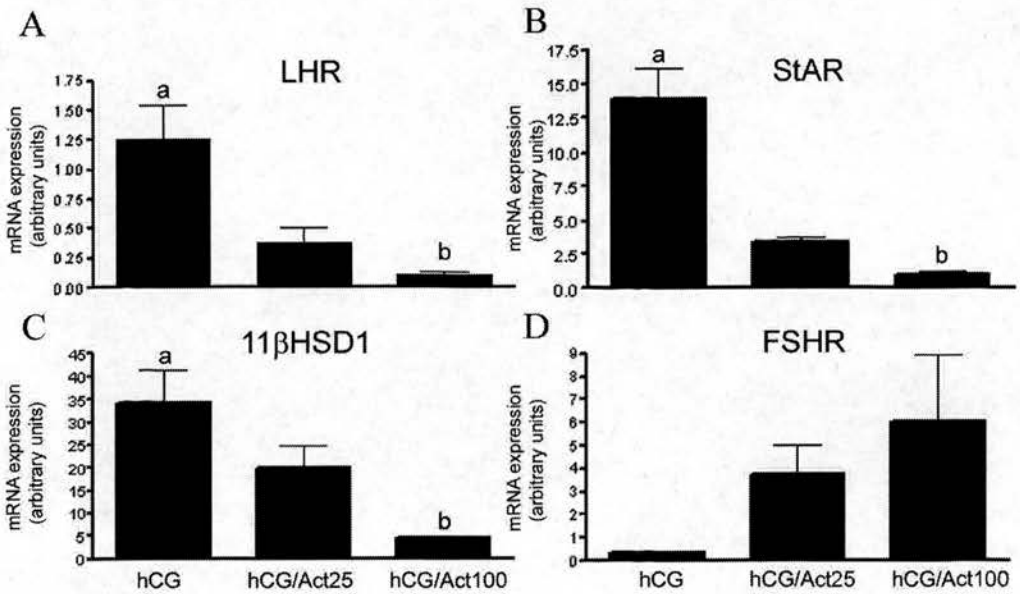


Figure 5.4 Activin acts in a dose-dependent manner to attenuate the response of hCG. **A,** Activin A attenuated the hCG effect on LHR ($p<0.01$, Kruskal-Wallis), **B,** StAR ($p<0.001$, Kruskal-Wallis) and **C,** 11βHSD1 ($p<0.01$, ANOVA) by minimising hCG action in a dose-dependent manner in primary cultures of luteinised granulosa cells. **D,** The opposite trend was observed with the FSHR whereby expression tended to increase with the dose of activin A.

5.3.5 The likely effects of activin A and hCG on activin A action.

Activin action can be inhibited at several levels including synthesis, secretion and reception. Increasing the expression of the inhibin α subunit will increase inhibin A and reduce activin A synthesis. Follistatin will bind to activin A and inhibit its action and the expression of β-glycan facilitates inhibin A inhibition of activin action at a receptor level. The addition of hCG increased the expression in inhibin α subunit ($p<0.01$), and its β-glycan receptor ($p<0.001$) (Figure 5.5). Activin A significantly ($p<0.01$) lowered the hCG-stimulated expression of follistatin and tended to increase the expression of the activin βA subunit (Figure 5.5).

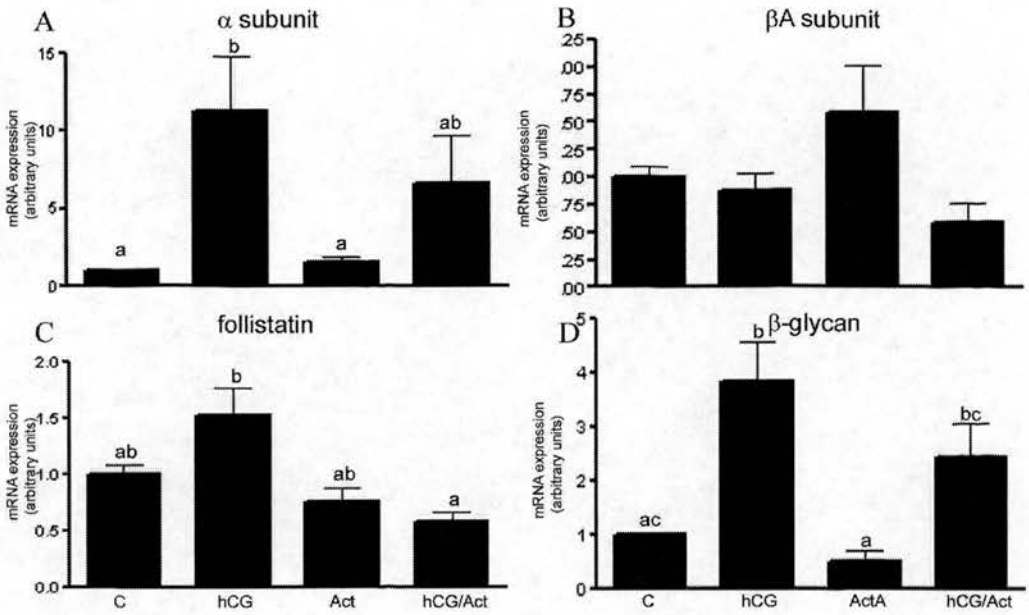


Figure 5.5 The likely effect of hCG and activin A on activin action. **A,** In primary cultures of luteinised granulosa cells, hCG up-regulated the expression of the inhibin α subunit ($p<0.01$, ANOVA) whilst activin A tended to attenuate this effect. **B,** HCG alone had no effect upon the expression of the common βA subunit ($p>0.05$, ANOVA) whilst activin A tended to up-regulate it. The activin A effect was dimished in cultures exposed to hCG and activin A concurrently. **C,** HCG tended to increase the expression of the activin inhibitor follistatin, whilst activin A alone had no effect, it reduced the hCG effect in co-treatments ($p<0.01$, ANOVA). **D,** The inhibin co-receptor β-glycan was significantly augmented with hCG ($p<0.001$, ANOVA) and although activin A had no effect on receptor expression, co-treatment with hCG tended to decrease the hCG response.

5.4 Discussion

The current study has investigated the effects of activin A and hCG on human luteinised granulosa cells *in vitro*. Already, studies from this thesis have suggested that hCG and activin have opposing effects on the regulation of tissue remodelling associated with luteolysis (Chapter 3; (Myers *et al.*, 2007a)). Herein, it is suggested that hCG and activin A have opposite effects on the luteinised granulosa cells involved in luteal formation. Activin A appears to reduce the luteinisation of luteinised granulosa cells so they have a more follicular phenotype, while hCG increases the luteinisation to a more luteal phenotype. The effect of activin on granulosa cells has been previously documented (Hutchinson *et al.*, 1987; Shukovski *et al.*, 1993; Shukovski *et al.*, 1991). However this investigation serves to highlight three concepts; 1) that hCG and activin A tend to have opposing actions on luteinised granulosa cells, 2) that activin A can reverse features of luteinisation once it has occurred and 3) that some of the effects of activin A are seen only in the presence of hCG. These studies increase our understanding of the role of activin in the folliculo-luteal transition.

Luteinisation of follicular granulosa cells into the granulosa-lutein cells of the corpus luteum is a key element of the folliculo-luteal transition. The formation of the corpus luteum itself involves a co-ordinated interplay of fundamental processes such as cell proliferation, cell differentiation, tissue remodelling and angiogenesis that is unparalleled in any other tissue in the adult mammal (Fraser and Duncan, 2005; Murphy, 2000; Stouffer, 2006). The mechanism and regulation of this process in not fully understood but it is driven by a complex co-ordination of steroid hormones, gonadotrophins, cytokines, growth factors and disparate paracrine signalling molecules. There are marked changes to steroidogenic cell functions during luteinisation. Granulosa cells become terminally differentiated, cell division ceases, the cells undergo marked hypertrophy and there are marked changes in the expression of different molecules including steroidogenic enzymes and hormone receptors (Murphy, 2000; Stocco *et al.*, 2007). The proximal event that initiates ovulation and luteinisation is the pre-ovulatory LH gonadotrophin surge (Filicori, 1999; Murphy, 2000), mimicked by hCG, acting via LH/hCG receptors. LH/hCG may directly induce the disparate and marked changes within the follicular granulosa cells phenotype that are necessary for appropriate luteal cell function whilst simultaneously withdrawing factors that are thought to inhibit luteinisation.

Inhibitors of luteinisation have been reported in many studies and indeed candidate molecules have been suggested. Indeed it is clear that luteinisation can occur in the absence of LH/hCG when granulosa cells are removed from the follicular environment. The physical presence of the oocyte has been reported to inhibit luteinisation in many species, for example, the mouse (Vanderhyden *et al.*, 1993), pig (Coskun *et al.*, 1995) and human (Seifer *et al.*, 1996) with candidate factor(s) acting in a paracrine signalling fashion from the oocyte to the granulosa cells. Furthermore, factor(s) in the follicular fluid of small, but not large follicles, have also proved to be inhibitory (Channing *et al.*, 1978; Ledwitz-Rigby *et al.*, 1977; Murphy, 2000). The nature of these molecules is not entirely clear but it is likely that members of the TGF-β family are involved (Glister *et al.*, 2005; Shimasaki *et al.*, 1999).

One member of the TGF-β superfamily that does have a role in follicular granulosa cell function is activin A. Activin A, is locally produced by follicles, predominantly smaller follicles, during their growth phase (Knight and Glister, 2006). As a follicle matures, activin continues to be produced but the follicular microenvironment becomes less activin dominant and more inhibin/follistatin dominant (Hillier, 1991b; Roberts *et al.*, 1993; Schneyer *et al.*, 2000; Yamoto *et al.*, 1992). However, it is well established that activins are essential for follicular granulosa cell proliferation, FSH receptor regulation, FSH-induced aromatase expression, decreased theca cell androgen production, and increased oocyte maturation (Knight and Glister, 2006). While a trophic role in the follicle is apparent, the role for activin A in the corpus luteum is less clear. Indeed, in women plasma concentrations of activin A tend to increase towards luteolysis (Muttukrishna *et al.*, 1996) and its local actions appear to facilitate luteolysis (Chapter 3; (Myers *et al.*, 2007a)). As activin A appears to have a positive role in the follicle and a negative role in the corpus luteum, activin action appears to be withdrawn at the follicular-luteal transition.

Activin action is tightly regulated at several different levels (Harrison *et al.*, 2005). The expression of the βA subunit itself is regulated (Hillier, 1991b; Roberts *et al.*, 1993) and in the presence of βA, up-regulation of the β subunit will push the synthesis of inhibin A rather than activin A. Inhibin A will also inhibit activin A at the level of its receptor by binding to β-glycan to complex with the activin receptor and inhibit activin action (Lewis *et al.*, 2000). In addition follistatin secretion is also regulated. Follistatin binds

irreversibly to activin to neutralise its bioactivity (Kogawa *et al.*, 1991; Nakamura *et al.*, 1990). The present chapter hypothesised that during ovulation one of the actions of LH/hCG was to change the follicular environment to inhibit activin action and this would facilitate luteinisation. This study was designed to analyse the effect of hCG on the expression of luteinisation and activin-associated genes in primary cultures of luteinised granulosa cells and investigate whether maintaining activin A action was able to inhibit or interfere with the effects of hCG in these already luteinised cells.

One of the effects of hCG is to promote the expression of genes involved in the synthesis of progesterone. Whilst follicular granulosa cells predominantly secrete oestradiol the granulosa-lutein cells are also able to secrete up to 100-fold greater amounts of progesterone. Indeed, it has been estimated that the human corpus luteum can secrete up to 40 mg of progesterone per day during the menstrual cycle (Lipsett, 1978). This is secondary to the increasing expression of LH receptors, StAR, $P450_{scc}$ and 3βHSD during luteinisation. The expression of StAR and 3βHSD in luteinised granulosa cells was increased by hCG while activin A could inhibit the increase in response to hCG in a dose dependent manner. Activins are well known to have inhibitory effects upon basal and hCG-induced progesterone secretion in dispersed cultures of granulosa-lutein cells in both the macaque (Brannian *et al.*, 1992) and in women (Di Simone *et al.*, 1994).

However activin A does not just interfere with hCG action as it has direct actions on luteinised granulosa cells. Activin A decreased LH receptor expression directly and increased FSH receptor expression in a dose dependent fashion using physiologically relevant concentrations. FSH and its seven-transmembrane receptor are essential for successful follicular development and granulosa cell function (Dierich *et al.*, 1998; Kumar *et al.*, 1997), however once granulosa cells have differentiated into those of the corpus luteum FSH receptors in the human corpus luteum are minimally expressed (McNeilly *et al.*, 1980). Studies in rats have shown that FSH receptor expression is down regulated during luteinisation (Camp *et al.*, 1991) whilst *in vitro* cell studies have shown that hCG treatment completely abolishes the receptor expression (Nakamura *et al.*, 1991). As well as inhibiting hCG-induced progesterone synthesis activin A directly induced a more follicular phenotype on already luteinised granulosa cells. These results highlight the importance of adequately suppressing active activin action during luteinisation and

maintaining this suppression in the functional luteal phase so that progesterone synthesis is not interrupted.

It is not just the progesterone synthesis pathway that changes during luteinisation. Another change is in the metabolism of cortisol by steroidogenic cells during the follicular and luteal phases. The 11βHSD enzymes control cortisol metabolism by their type 1 (that tends to generate cortisol) and type 2 (that tends to inactivate cortisol) isoforms. Numerous studies have shown clear evidence that there is a switch in 11βHSD isoforms associated with the follicular-luteal transition (Michael *et al.*, 1997; Tetsuka *et al.*, 1997; Thurston *et al.*, 2003; Yong *et al.*, 2000). This has been further confirmed in Chapter 4, whereby the switch in women at a protein level and localised expression to the granulosa/granulosa-lutein cells of the ovary. Type 1 11βHSD protein is not found in antral follicular granulosa cells whilst type 2 11βHSD is abundant. Similar to the mRNA transcripts patterns observed in the human corpus luteum (Chapter 4; (Myers *et al.*, 2007b)), both 11βHSD isoforms are present in granulosa-lutein cells, however there has been a marked switch in the predominant isoform to type 1 with only low levels of type 2 still detectable. The current study demonstrates that activin A suppresses hCG actions on type 1 11βHSD expression, whilst hCG attenuates activin actions on type 2 11βHSD. These responses in luteinised granulosa cells give important insights into the switch in 11βHSD isoforms that consequently predict the local cortisol environment in the follicular or luteal phase and highlight the regulation of 11βHSD expression, and the opposing actions of hCG and activin, during luteinisation.

The opposing action of hCG and activin A during luteinisation can also be seen when the expression of ERα is investigated. Although the corpus luteum of women and primates express oestradiol receptors in the form of ERβ (Duffy *et al.*, 2000; Saunders *et al.*, 2000) the steroidogenic cells of the primate corpus luteum, unlike follicular granulosa cells, are generally thought not to express ERα (Chandrasekher *et al.*, 1994; Iwai *et al.*, 1990). Herein ERα has been immunolocalised to the nuclei of granulosa cells of antral follicles and clearly absent in the granulosa-lutein cells of the corpus luteum. This is most likely due to the preovulatory LH surge which may be involved in switching off follicular ERα expression as the granulosa cells differentiate into granulosa-lutein cells of the corpus luteum. Results from cell culture in the present study suggest that ERα is differentially regulated by activin A and hCG in luteinised granulosa cells as without activin A in the

system, hCG down regulated ERα expression. These findings are consistent with work involving the oestrogen receptor null mice that suggest that ERα, unlike ERβ, tends not to facilitate granulosa cell differentiation in the peri-ovulatory period (Couse *et al.*, 2005). The role of LH/hCG in luteinisation is clear and facilitated by the reduction in activin action.

It is important to inhibit activin action during luteinisation. This is evident from the present study whereby the effect of activin A alone or in inhibiting hCG action is dose dependent. The effect of hCG in luteinised granulosa cells is to continue to remove activin action. In corpora lutea and luteal cells *in vitro* (Illingworth *et al.*, 1996; Muttukrishna *et al.*, 1997) and in cultured luteinised granulosa cells (Myers *et al.*, 2007a) hCG increases inhibin A expression. Furthermore, hCG up-regulated the β subunit and activin A itself inhibited this response. Similarly hCG increases β-glycan expression and this is attenuated in the presence of activin. Indeed hCG up-regulates follistatin (Myers *et al.*, 2007a) and activin inhibits this effect. As activin A itself seems to be involved in promoting activin action it is likely that continued LH/hCG effects during luteinisation, and in the corpus luteum, by inhibiting activin, would further reduce activin action in a feedback loop. As LH action seems to be abrogated in the corpus luteum during luteolysis (McNeilly *et al.*, 1980; Messinis, 1997) the inhibition of activin action will be reduced and activin may feed-forward to increase its action and facilitate a role in luteolysis (Myers *et al.*, 2007a) that will be self-limiting as steroidogenic cells are removed from the ovary (Yuan and Giudice, 1997).

One of the features of luteinisation is terminal differentiation and the cessation of cell division. The effect of activin A on the potential of the luteinised granulosa cells to divide has not yet been investigated. However by showing that activin A can induce a more follicular granulosa cell phenotype of these cells it may be suggested that the differentiation state of these cells may be more plastic that expected. A marker of the cell cycle, such as cyclin D2 is up-regulated by FSH and down-regulated by LH in granulosa cells (Sicinski *et al.*, 1996). Conversely, $p27^{kip-1}$, a cdk inhibitor protein that inhibits entry into the cell cycle and maintains differentiation (Robker and Richards, 1998) is known to be expressed in granulosa cells in response to LH and along with $p21^{cip1}$ is maintained at high levels in (mouse) corpora lutea (Deng *et al.*, 1995; Robker and Richards, 1998). Furthermore, mice deficient in the $p27^{kip-1}$ gene show that corpora lutea formed after

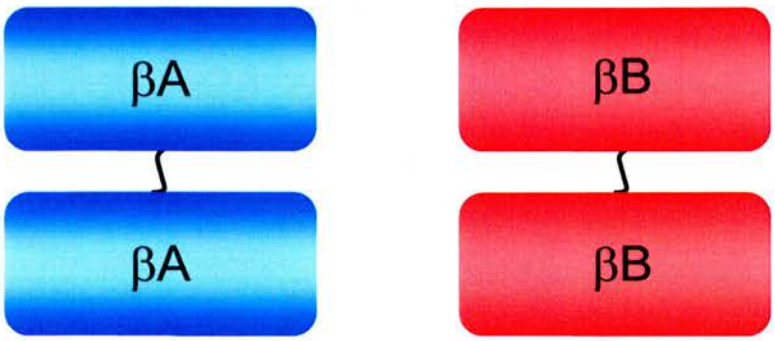
luteinisation do not stop proliferating (Tong *et al.*, 1998). It could by postulated that activin A may be able to facilitate cell division in luteinised cells just as it can in FSH-treated unluteinised granulosa cells (Kumar *et al.*, 1997; Li *et al.*, 1995; Matzuk *et al.*, 1996; Ogawa *et al.*, 2003). Indeed a preliminary report suggested increased cell numbers in cultures of human luteinised granulosa cells grown in the presence of activin A (Rabinovici *et al.*, 1990) although it should be noted that this study only reports an increase in cell number and it cannot be excluded that the increase in number may be attributed to other contaminating cell types (ie fibroblasts, endothelial cells) (Duncan *et al.*, 2005b) that are not terminally differentiated. It is clear from both *in vivo* and *in vitro* studies that activins do promote follicular granulosa cells proliferation, however whether or not they can 'rescue' or 'reverse' the luteinised granulosa non-dividing phenotype type remains unknown.

The identification of activins having a role in luteinisation is not novel. This research however focuses on the luteinised granulosa cell as a luteal cell that has already been luteinised by hCG *in vivo*. In that circumstance activin A action appears to be inhibited. If activin A is put back in the system it can inhibit hCG action and has a direct anti-luteinisation effect. It seems that removal of activin is important to allow formation of the corpus luteum. As increased activin A in the late-luteal phase can facilitate removal of the corpus luteum it appears that activin A is anti-luteal at both extremes of the luteal lifespan (Myers *et al.*, 2007a). The Smad4 conditional knockout mouse model is another system that provides evidence for activin A as an inhibitor of luteinisation. These mice demonstrate a loss of both TGF-β and activin signalling with targeted disruption of the common Smad 4 in ovarian granulosa cells (Pangas *et al.*, 2006). Premature luteinisation of the follicular granulosa cells was one of the key phenotypes of this mouse, which was clearly evident by an up-regulation of genes associated with luteinisation. Luteal markers such as the LH receptor, StAR, $P450_{scc}$ and 3βHSD were all up regulated in the absence of activin signalling, whilst FSHR was slightly down regulated. Indeed disrupted activin A action in mature ovaries of the mouse resulted in an excess of corpora lutea (Pangas *et al.*, 2007).

In summary, this chapter has presented evidence that activin A and hCG have opposing molecular effects upon genes involved in the follicular-luteal transition in the ovary. These results suggest that removal from, or at least suppression of activin in, the system is

very important for successful luteal function. Furthermore, local activin action may be detrimental to luteal function and structure and therefore successful luteal function requires inhibition of activin action whereas luteal involution is facilitated by an increase in activin activity during luteolysis (Myers *et al.*, 2007a).

6 Activin A vs. activin B: is there a role for activin B in the human corpus luteum?



Activin A Activin B

One of the major findings of this thesis has been characterisation of activin A as a luteolysin in the human corpus luteum. For the first time, this thesis provides good evidence to suggest a functional role for activin A in the human corpus luteum. Although we are beginning to understand more about this activin form, less is known about activin B. Therefore, the focus of Chapter 6 is to investigate a potential role for activin B in the human corpus luteum and to determine whether it has similar or different paracrine or endocrine interactions to activin A.

(The basic molecular structures of the activin A and activin B isoforms)

6.1 Introduction

Activins and inhibins are members of the TGF-β superfamily that have been well characterised for their roles in reproductive development. Structurally related, yet functionally diverse the activins (β:β) and inhibins (α:β) are composed of mature subunit chains linked together by disulphide bonds. The β-subunit expression determines the activin/inhibin subtype, with βA forming either inhibin A (α:βA) or activin A (βA:βA) and the βB chain conferring inhibin B (α:βB) or activin B (βB:βB). Futhermore, activin AB (βA:βB) also exists. Initially discovered for their opposing abilities to regulate pituitary FSH, much work over the past decades has focused upon the disparate roles of these glycoproteins.

Assays for inhibin and activin have greatly increased our knowledge of the expression patterns of these glycoproteins across the ovarian cycle. These precise and replicable results have clearly shown the diverse expression patterns of the inhibin A, inhibin B, activin A and activin AB in naturally cycling women (Evans *et al.*, 1997; Groome *et al.*, 1994; Groome *et al.*, 1996; Groome *et al.*, 1995; Knight and Muttukrishna, 1994). Such assays have proved successful by utilising panels of monoclonal antibodies to synthetic peptide immunogens that were used to construct enzyme-linked immunosorbent assays (ELISA) (Lockwood *et al.*, 1998b). Unfortunately, due to the lack of a readily available, reliable activin B assay, this protein remains less characterised across the ovarian cycle in the current literature.

However with the information from invaluable previous work detailing the protein expression patterns of inhibin A, inhibin B and activin A across the menstrual cycle in women, coupled with interventional *in vitro* work, well-informed hypotheses can be made. With the reported low concentrations of inhibin B during the luteal phase (Illingworth *et al.*, 1996), and the localisation of βB in the corpus luteum, the present chapter hypothesised that activin B may indeed be a product of the human corpus luteum and potentially have an endocrine or paracrine role. More recently, a mechanistic role for activin A was suggestesd during human luteolysis (Myers *et al.*, 2007a). Already activin A and activin B have been shown to exhibit both differential (Matzuk *et al.*, 1995; Vassalli *et al.*, 1994) yet overlapping roles within the ovary (Brown *et al.*, 2000). Therefore the aim of the current study was to seek out a role for activin B in the human corpus luteum and if so, to determine if it is different to that of activin A. This chapter

documents preliminary results with an Oxford Brookes University-developed activin B assay that is still currently under optimisation. However, a prelude into some of the future work and working hypotheses will give an insight into potential roles for activin B in the human corpus luteum.

6.1.1 Aims

To identify and localise the βB subunit of activin B in the human corpus luteum

To assess the gene expression and regulation of the βB subunit in primary cultures of luteinised granulosa cells

To utilise a reliable ELISA assay for the activin B protein which does not cross-react with the βA subunit such that activin B is easily distinguishable from activin A and inhibin A.

To utilise this assay for *in vitro* experiments to further understand potential endocrine and paracrine signalling molecules that may have effects upon activin B secretion.

To investigate a possible paracrine role for activin B in the human corpus luteum by assessing the expression and activity of the proteolytic enzyme MMP-2

To determine if activin A and activin B have differential role in the human corpus luteum

6.2 Materials and Methods

6.2.1 Collection of tissues and cells

Ovarian/corpora lutea tissue (n=3) and cells were obtained as described in section 2.1.2 2.1.3. Human endometrial tissue (n=3) was obtained at the time of collection of the corpus luteum and dated in the same manner (section 2.1.2.2) (Duncan *et al.*, 1996b). Informed consent was obtained from all patients and the study was approved by the research ethics committee.

6.2.2 Isolation of human luteinised granulosa cells and derivation of fibroblast-like cells

Isolation of luteinised granulosa cells using Percoll gradient centrifugation was carried out as described in section 2.1.3. Twenty-four well cell culture plates were precoated with 25 µl of matrigel (BD Biosciences) and 100,000 viable cells per well were plated in 1 ml of serum-free media.

Fibroblast-like cells were obtained from prolonged cultures of follicular aspirates as described in section 2.1.4 and 60,000 cells per well were plated in 1 ml of 10% FBS culture medium on 24-well cell culture plates.

6.2.3 Primary cell culture treatments

Each pooled experiment for the following treatments was carried out at least 3 times to avoid biological bias.

6.2.3.1 Activin A and hCG treatments in primary cultures of luteinised granulosa cells

Pooled luteinised granulosa cells in serum-free medium were refreshed with serum-free culture medium every 2 days after until day five when they were treated with the combinations of human recombinant activin A (R&D Systems) or a maximally stimulating dose of hCG (Serono). The following six treatments were added to luteinised

granulosa cells; 1) 0.1% BSA in sterile PBS; 0.1% BSA with 2) 100 hCG; 3) 25 ng/ml activin A; 4) 100 ng/ml activin A; 5) 25 ng/ml activin A with 100 ng/ml hCG; and 6) 100 ng/ml activin A with 100 ng/ml hCG. Each pooled experiment for the following treatments was carried out at least three times to avoid biological bias.

6.2.3.2 Manipulation of hCG in prolonged cultures of luteinised granulosa cells

In order to mimic the luteal phase in primary cell culture, luteinised granulosa cells were plated as described above and grown for 12 days as described in section 2.1.3.1. Briefly, cells were stimulated with low dose hCG (1 ng/ml) with LDL (50 mg/l) beginning on day 2 and this was repeated every 2nd day until day 7 when treatments were replaced with maximal doses of 100 ng/ml hCG/LDL or LDL alone. Cells were analysed after seven days with hCG and on day 12 in the presence or absence of hCG to mimic the progesterone secretion profile of late-luteal and luteal rescue stages respectively (Duncan *et al.*, 2005a).

6.2.3.3 Treatment of fibroblast-like cells with activin B

Cultures of fibroblast-like cells (n=3, experiments) were plated as above and after 6 hours in serum-free culture, the medium was removed and replaced with medium containing recombinant activin B (R&D Systems, 25 ng/ml) or an equivalent amount of 0.1% BSA in carrier as a control. After 24 h the culture medium was collected for subsequent zymography and the cells were used for mRNA extraction.

6.2.4 Preparation of cDNA from corpora lutea and cultured cells

Messenger RNA was batch extracted from frozen human corpora lutea and reverse transcribed into cDNA using random hexamers as described in section 2.1.16. Cultured cell mRNA was extracted using RNeasy mini-spin columns after lysis by the addition of RNeasy lysis buffer (Qiagen). Lysates were frozen until processed as per manufacturers' protocols then DNase treated with on-column DNaseI (Qiagen) and quantified using the NanoDrop ND-1000 Spectrophometer (NanoDrop Technologies). Messenger RNA was then reverse transcribed into cDNA using random hexamers (Applied Biosystems).

6.2.5 Quantitative analysis of gene expression by RT-PCR

Quantitative real-time PCR was carried out on the ABI PRISM 7900 heat-cycler sequence detection system (Applied Biosystems) using specific primers and probes (Eurogentec) for the βB subunit and levels were related to a ribosomal 18S internal control (Applied Biosystems). All samples were performed in duplicate and a relative comparison was made to an appropriate tissue control tissue cDNA. Primer and probes are listed in Table 6.1.

Table 6.1 Table of all primer/probe sequences used for Taqman quantitative RT-PCR and reference obtained from.

Gene	Fwd primer 5'-3'	Rev primer 5'-3'	Probe 5'-FAM-TAMRA-3'
Inhibin βB subunit M13437	CCTGGGATCCTTCGTGCT T	GTTGTGTCCTTTCTGGGTCT CTTT	CCTGTCCTTCCATGC CCTTGTCGA (Casagrandi *et al.*, 2003)
MMP-2 NM_004530	TTCCTGGGCAACAAATATG AGA	TGGTCGCACACCACATCTTT	AGCGCCGGCCGCAGTGA (Jordan *et al.*, 2004)
Smad 7 NM_005904	GTGGCATACTGGGAGGAG AA	TTGTTGTCCGAATTGAGCTG	TACTGTGTCCAGGAGCCC TCTCTGG (Springer *et al.*, 2004)

6.2.6 Gelatin zymography

Gelatin zymography was performed on the culture medium from activin B treated fibroblast-like cell cultures as described in section 2.1.11.

6.2.7 Measurement of activin B in primary cell culture medium

Activin B concentrations were measured in primary cell culture samples with a newly developed activin B ELISA assay. Optimised and performed by Helen Ludlow and Nigel Groome at Oxford Brookes University (Headington, Oxford), this assay uses the newly developed 46A/F βB antibody for both capture and detection. This assay is still in developmental stages and consequently the results presented in this thesis are preliminary. The observed patterns of the assay are most likely true, however further validation and optimisation of the assay will further confirm specificity and validity of actual values obtained. This assay is currently being optimised for serum samples which

will prove extremely useful for further characterising the concentrations of activin/inhibins over the human ovarian cycle.

6.2.8 Immunohistochemistry

To allow staining with the βB antibody in a controlled and easily repeatable manner, the Bond-X automated immunostaining machine (Vision Biosystems, Newcastle, UK) was utilised. Human ovary paraffin sections were cut, deparaffinised, rehydrated and subjected to antigen retrieval in 0.01 M citrate buffer as described in sections 2.1.9.2, 2.1.9.3 and 2.1.9.3 before being place on the Bond-X machine. This method on the Bond-X automated machine utilises a specific polymer high contrast program. Briefly, slides were peroxidase blocked for 5 min, incubated for 2 h with the primary antibody diluted 1 in 500 in the diluent supplied and then incubated with the post-primary reagent for 15 min. Control sections were incubated with diluent alone or mouse IgG1 protein (Serotec) diluted to the same concentration as primary antibody in supplied diluent to confirm antibody specificity. Sections were then incubated with the polymer reagent for 15 min to increase sensitivity of detection prior to DAB detection for 10 min. Sections were counterstained in haematoxylin for 5 min. Slides were then removed from the machine and dehydrated and mounted using Pertex.

6.2.9 Statistical analysis

Statistical analyses used are highlighted in the Figure legends. Parametric statistics were used if the data was normally distributed with similar standard deviations. Groups were analysed by ANOVA with Bonferroni pairwise comparisons; if the data was not normally distributed non-parametric statistics were used. Groups were analysed by Kruskal-Wallis test with Dunn's Multiple Comparison Test. A paired t-test was used when treatment and control samples were analysed. Differences were considered significant at $p<0.05$ level.

6.3 Results

6.3.1 Localisation of the activin/inhibin βB subunit in various reproductive tissues using the 46A/F antibody.

With the newly developed mouse monoclonal βB antibody (46A/F) (kind gift from Helen Ludlow and Nigel Groome, Oxford Brookes University), localisation of the βB subunit in various reproductive tissues was explored. Unlike the previous monoclonal anti-βB subunit antibody (C5) which had 0.5% cross reactivity with the βA subunit, the new monoclonal anti- βB (46A/F) antibody has only 0.0006% cross-reactivity with activin A subunits (Ludlow *et al.,* personal communication). Localisation of the βB subunit in ovarian and endometrial tissue was investigated using the improved antibody. Positive staining was observed in all sections assessed. In the secretory staged endometrial tissue (Figure 6.1A) staining was clearly noted in the glandular endothelium (notably the luminal epithelium) and to a lesser extent the surrounding stromal tissue compartments. This staining concurred with other publications (Jones *et al.*, 2000b; Mylonas *et al.*, 2004) that used other βB antibodies in secretory endometrium, however the staining in the current study using the 46A/F antibody demonstrated much cleaner, more specific staining. This is most likely due to the low cross-reactivity of the 46A/F antibody with the βA compared to that of the other antibodies. Localisation of the βB subunit was also evident in the ovary of follicle of different developmental stages (Figure 6.1B-C). Subunit expression was clearly evident in the pre-granulosa cells and oocyte of the primordial follicle (Figure 6.1A) and many cell types of an antral follicle. Specific staining was confirmed with a mouse IgG1 negative control (Figure 6.1D inset). It is also interesting to note that when used under the exact same conditions (including antibody concentration), localisation of the C5 βB antibody on a section with an antral follicle, had a very dirty, less specific staining phenotype compared to that of the 46A/F antibody (Figure 6.1C-D) that was much cleaner. Presumably, this is a result of the greater incidence of cross-reactivity of the C5 antibody with the βA subunit, which is also localised to these cells types and consequently amplifies the observed detection signal. Consistent with the rest of this chapter the βB subunit, utilising the newly developed antibody, was detected in the cells of the human corpus luteum (Figure 6.1E,F). At low power this was most notable in the steroidogenic cells however at a higher magnification it became evident that βB was expressed in both granulosa-lutein and theca-lutein cells

and to a much lesser extent the surrounding stromal area. A negative control section (mouse IgG1) demonstrated that this staining was specific to these cells types.

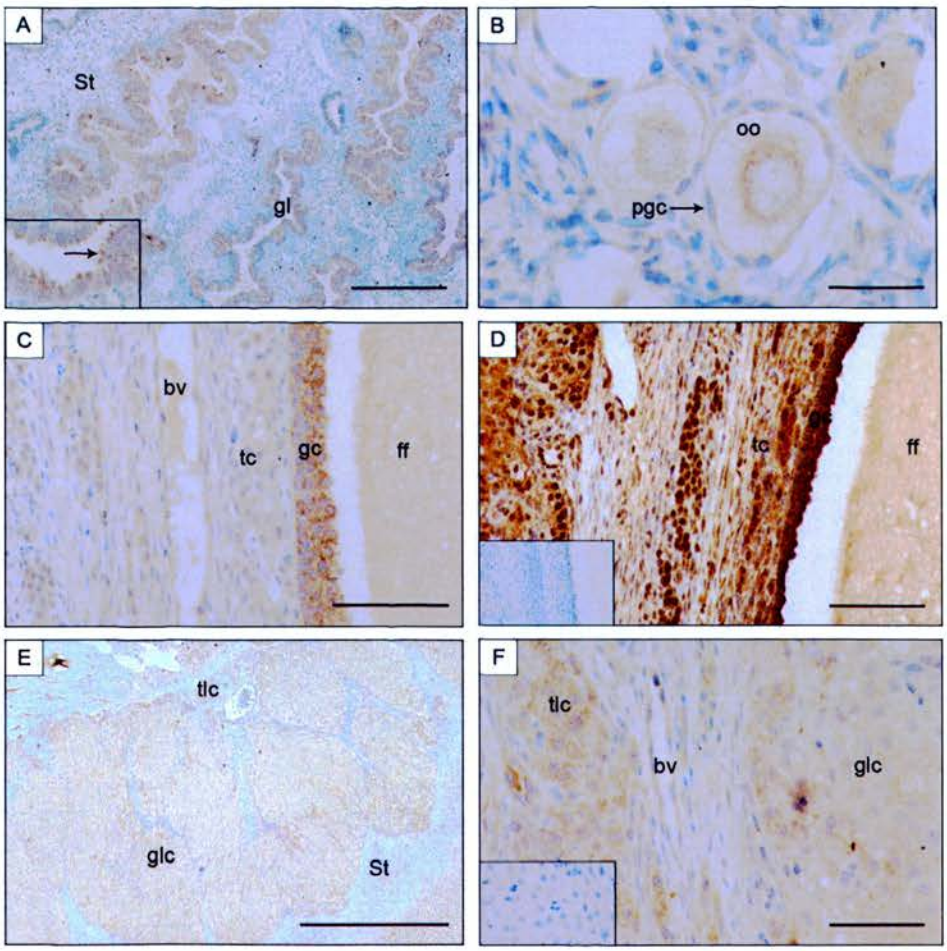


Figure 6.1 Immunohistolocalisation of the βB subunit in the human endometrium and ovary. **A,** Human endometrium in the secretory phase shows βB localised to the glandular epithelium (gl), particularly in the luminal side (pointing arrow on inset), and to a lesser extent in the stromal (St) compartment. **B,** The βB subunit can be found in the smallest of follicles, the primordial which demonstrated positive staining around the nucleus of the oocyte (oo) and in the cytoplasm of the surrounding squamous pregranulosa cells (pgc). **C,** βB is present in the many cell types of an antral follicle with the strongest staining in the granulosa cells (gc). Surrounding thecal cells (tc) are also positive for βB and indeed a blood vessel (bv) within this layer also shows localisation of the activin/inhibin subunit. As expected, the βB subunit is also evident in the follicular fluid (ff) of the antral cavity. **D,** Interestingly, replacing the 46A/F antibody with the C5 antibody in the exact same conditions results in staining in the same cell types of the antral follicle, however to a much greater extent. Inset demonstrates that staining of the follicle is specific as the IgG1 control is negative for panels C and D. **E,** The corpus luteum exhibits positive staining for the βB subunit. At low power βB is clearly evident in the granulosa-lutein cells (glc). **F,** At high power however it is clearly evident that βB is also localised to the adjacent theca-lutein cells (tlc) and the endothelial cells of a blood vessel (bv) in the surrounding stromal (St) area. Scale bars, A=200 μm, B=30 μm, C=50 μm D=100 μm E=1000 μm F=50 μm

6.3.2 Quantification of the α and βB subunits of activin and inhibin B across the human luteal phase

To investigate the changes in the α chain of inhibin and the βB chain of inhibin B and activin B across the luteal phase, carefully dated human corpora lutea were investigated using quantitative RT-PCR (Figure 6.2). Expression of the α subunit remained constant over the luteal phase (Figure 6.2A) however in the presence of exogenous hCG, during luteal rescue, expression was markedly increased ($p<0.05$, Kruskal-Wallis). The same was true for the βB subunit where by expression also remained constant across the normal luteal phase, however the opposite occurred during luteal rescue whereby βB tended to decline (Figure 6.2B). The marked increase in α subunit suggests that during maternal recognition of pregnancy the increase in α and decrease in βB suggests that the potential for activin B synthesis is reduced.

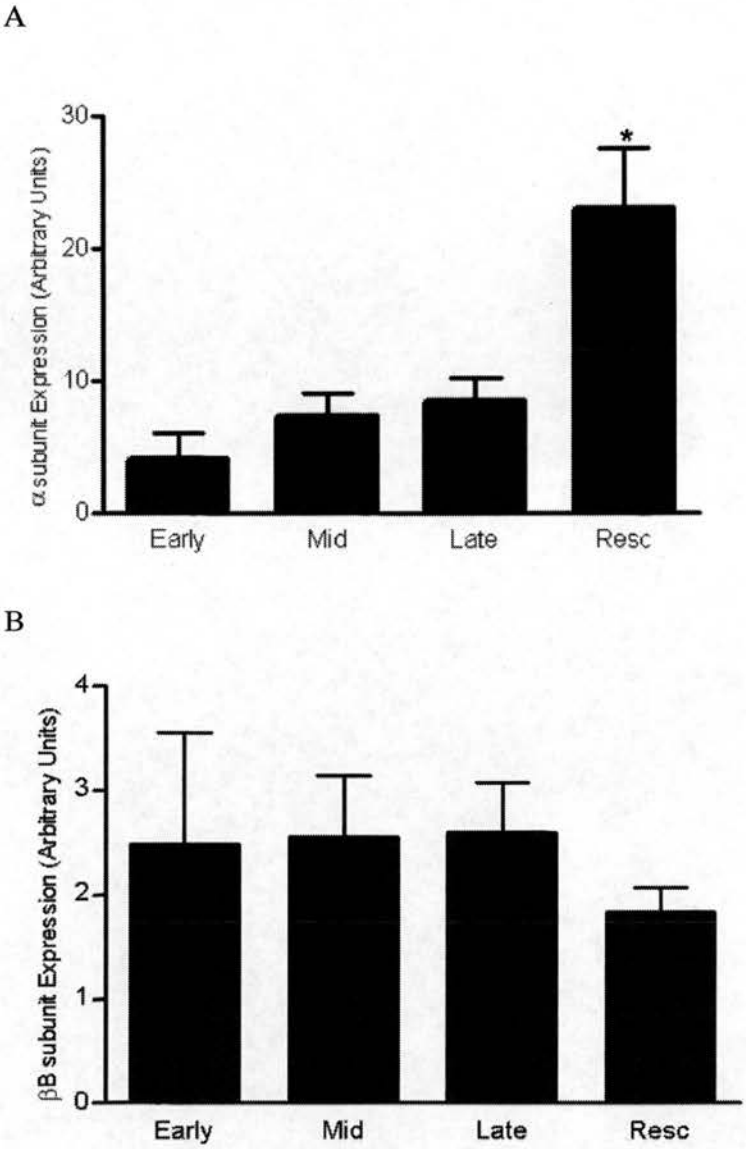


Figure 6.2 Expression of the α chain of inhibin and the βB chain common to both activin B and inhibin B across the luteal phase in the human corpus luteum. **A,** Real-time quantitative RT-PCR shows the α subunit is significantly up regulated during luteal rescue ($p<0.05$, Kruskal-Wallis). **B,** Whilst the βB subunit tends to decrease at this stage it did not reach significance.

6.3.3 Expression of the βB subunit and activin B concentrations in the 'artificial' luteal cycle mirror the human luteal phase

Primary cultures of luteinised granulosa cells treated to mimic the luteal phase demonstrate that both the βB subunit (Figure 6.3A) and concentrations of activin B (Figure 6.3A) (range 7-26 pg/ml) as detected with the newly developed ELISA, mirror

the mRNA expression patterns observed with the βB subunit over the human luteal phase in Figure 6.2B. These results suggest that activin B is regulated by gonadotrophins and one of the roles of hCG during luteal rescue may be to decrease activin B.

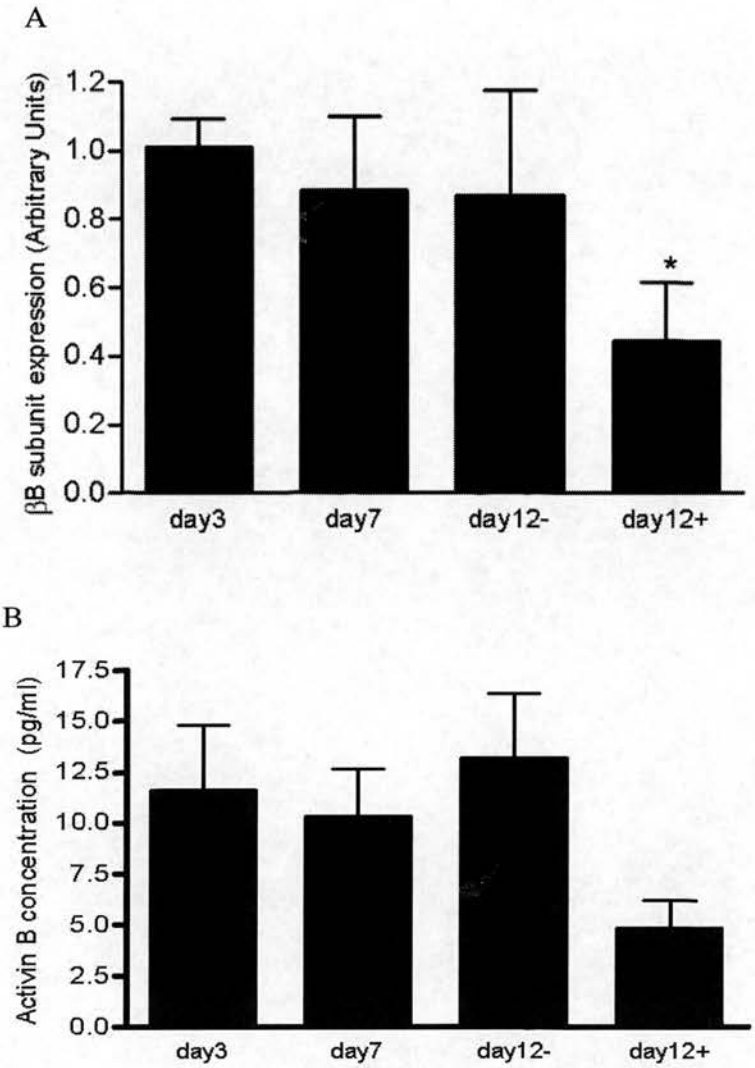


Figure 6.3 Chronic manipulation with hCG in cultures of luteinised granulosa cells designed to mimic the luteal phase involved low dose hCG stimulation until day 7, which was either replaced by maximal dose of the hCG on day 12 (+) or removal of hCG on day 12 (-). **A,** Expression of the βB subunit in primary cell culture did not change with hCG withdrawal, however chronic stimulation with hCG significantly decreased mRNA expression ($p<0.05$, Kruskal-Wallis). **B,** Concentrations of activin B mirror the observed pattern of subunit expression in primary cell culture.

6.3.4 Expression of the βB subunit is differentially regulated by activin A and hCG in primary cultures of luteinised granulosa cells

HCG and activin A had differential effects upon the βB subunit *in vitro*. Activin A up regulated the mRNA expression of the βB subunit at 25 and 100 ng/ml ($p<0.01$, Kruskal-Wallis). HCG alone tended to decrease βB subunit expression, and when added simultaneously with activin A treatments it was able to inhibit the activin effect.

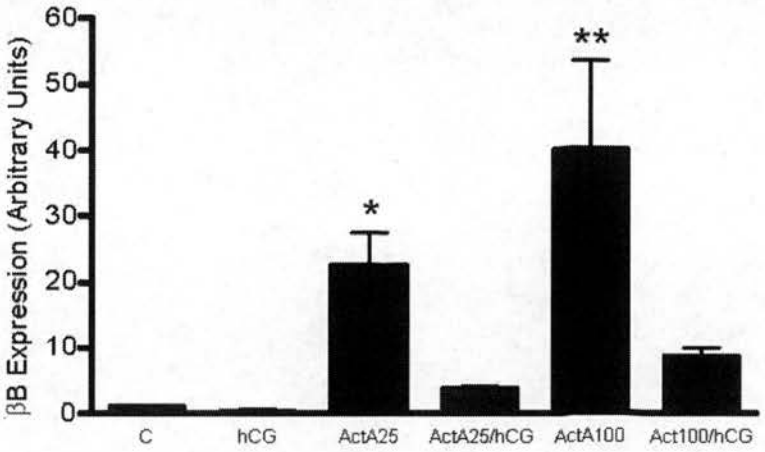


Figure 6.4 βB subunit mRNA expression is differentially regulated by hCG and activin A in primary cultures of luteinised granulosa cells. Activin A significantly up regulated βB expression at both low ($p<0.01$, Kruskal-Wallis) and high ($p<0.01$, Kruskal-Wallis) doses, whilst hCG tended to attenuate this effect.

6.3.5 Activin B concentrations in activin A and hCG treatments of luteinised granulosa cells

Unlike the mRNA expression, activin B concentrations (in the range of 7-100 pg/ml) were not as tightly regulated by activin A and hCG. Low or high (25 or 100 ng/ml) activin A treatments (Figure 6.5) demonstrate trend to increase however this was not statistically significant. In contrast to chronic treatments with hCG (Figure 6.3), 24 h treatments did not downregulate activin B concentrations (Figure 6.5). This may be due to βB subunits still being present after only 24 h of gonadotrophin treatment, however after longer hCG treatment the activin B (as evident in chronic treatments) and indeed the inhibin B concentrations may decrease when the βB subunit production decreases. In any case, combination treatments with activin A and hCG demonstrate a trend to lower activin B concentrations although this was not statistically significant.

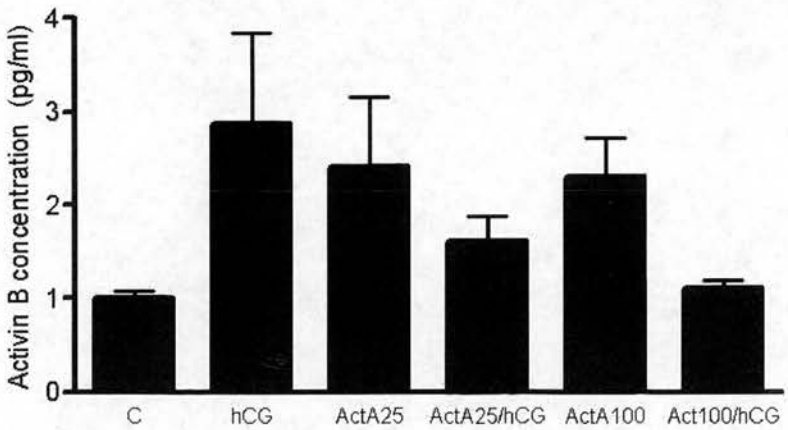


Figure 6.5 Activin B concentrations in primary cultures of luteinised granulosa cells. HCG and activin A do not regulate activin B in 24 h treatments.

6.3.6 Preliminary results suggest that activin B does not regulate the proteolytic MMP-2 expression or activity in fibroblast-like cells

Unlike activin A (Myers *et al.*, 2007a), preliminary results suggest that activin B does not regulate the expression or activity of MMP-2 in fibroblast-like cells. Consequently, activin B may not be involved in the tissue remodelling associated with luteolysis and may exhibit differential actions to that of activin A in the human corpus luteum.

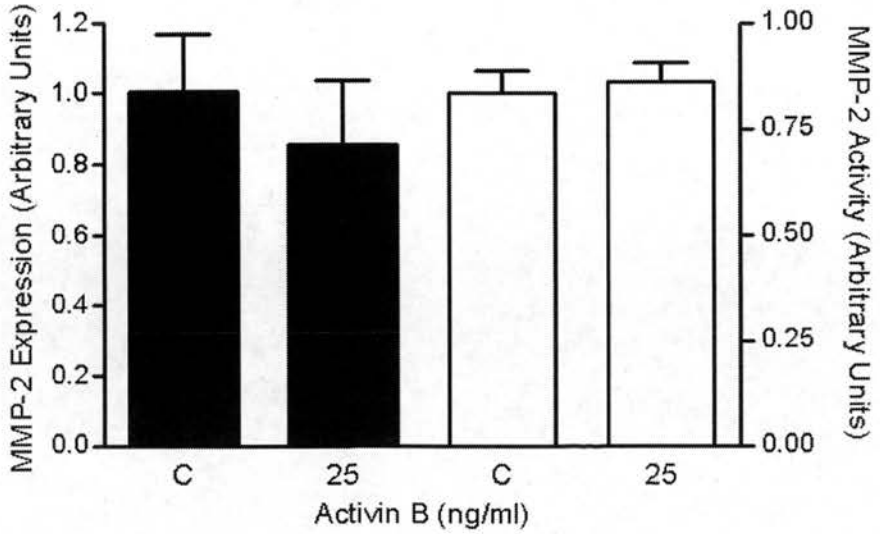
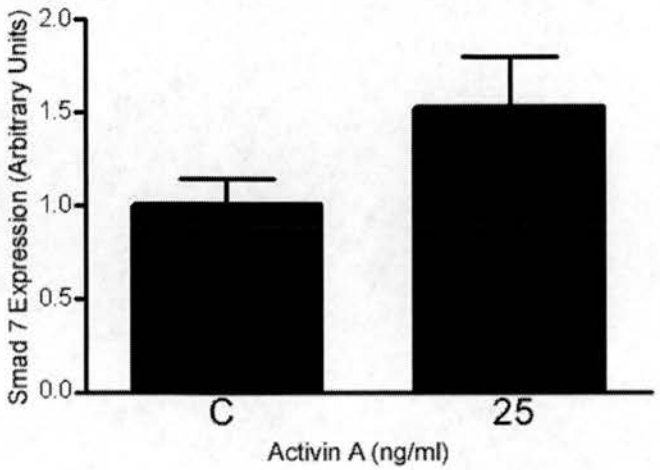


Figure 6.6 Activin B does not regulate MMP-2 in primary cultures of fibroblast-like cells. Relative MMP-2 expression using real-time quantitative RT-PCR, is shown in solid bars and MMP-2 activity, using gelatin zymography, is shown in open bars. MMP-2 expression ($p>0.05$, t-test) and activity ($p>0.05$, t-test) was not altered in fibroblast-like cells exposed to 25 ng/ml of activin B in culture (n=3 experiments).

6.3.7 Activin A and Activin B up regulate Smad 7 in fibroblast-like cells

As activin A and activin B are thought to act through the same receptor system this difference may be due to different binding at a receptor level or in modification affinities of signalling. Therefore the effect on inhibitory Smad 7 was analysed. Activin A and activin B have the same effect upon Smad 7 in fibroblast-like cells. Activin A tended to increase the expression of Smad 7 in fibroblast-like cells and preliminary studies in fibroblast-like cells show activin A exhibiting a similar effect. Smad 7 is indeed expressed in the human corpus luteum (data not shown), however this is yet to be fully documented.

A



B

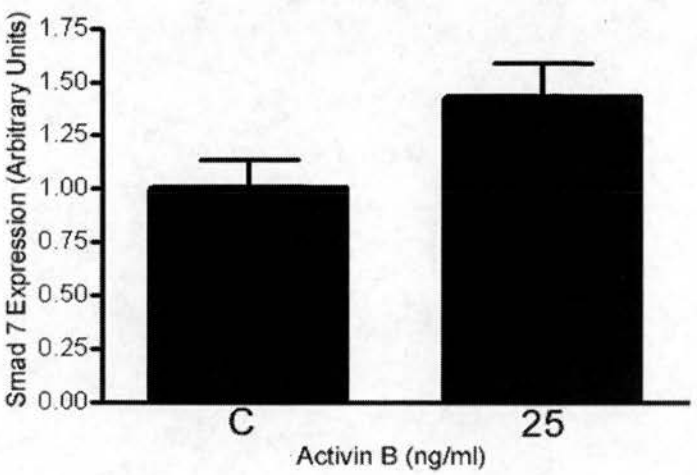


Figure 6.7 Activin A and activin B exhibit similar effects on the cytoplasmic protein Smad 7 in fibroblast-like cells. **A,** Whilst the up-regulation of Smad 7 in activin A treated cells was not significant ($p>0.05$, t-test) **B,** preliminary treatments ($n=2$) with activin B demonstrate that both activins have the effects on this Smad protein.

6.4 Discussion

The marked interest in activin/inhibin actions in ovarian biology over the past few decades has resulted in a surplus of information regarding these glycoproteins and related TGF-β superfamily members. From the initial isolation and purification over some 20 years ago, the discovery of the activins and inhibins has created a whole new niche in reproductive biology. This has indeed been the direct result the careful development of highly specific and sensitive two-site enzyme-linked immunoassays, for both *in vivo* and *in vitro* experimental conditions, coupled with the more recent transgenic mouse models of ovarian failure. Such studies have proven clear and definitive roles for activins and inhibins whilst simultaneously raising a plethora of new and exciting questions.

One of the current unanswered questions in activin/inhibin biology is a potential role for these proteins in luteal physiology. Expression of activin/inhibin subunits has been well explored in corpora lutea of many species (human, primate, rodent) however until very recently, a functional role had not yet been elucidated. Studies from the current thesis describe a potential paracrine role for activin A in the human corpus luteum, utilising mechanistic *in vitro* and observational *in vivo* approaches (Chapter 3; (Myers *et al.*, 2007a)). This chapter however raises a different question; does activin B have a similar or disparate role to that of activin A in the human corpus luteum? To date, a (functional) role for activin B in the human corpus luteum remains to be explored.

Activin B has been well characterised to the smaller pre-antral follicles in the many species studied, with the expression of the βB subunit reported to be in excess of the βA and α subunits (Roberts *et al.*, 1993). This suggests that activin B is preferentially produced/secreted by the smaller, less mature follicles (Liu *et al.*, 2001). Indeed inhibin B is a marker of small follicles (Bath *et al.*, 2003) and is detected at its highest concentrations in the plasma of women during their follicular phase (Groome *et al.*, 1996). Consistent with primary cell culture reports, induction with gonadotrophins (to mimic the preovulatory surge) increases βA and α subunit, whereas βB expression is reduced (Liu *et al.*, 2001; Tuuri *et al.*, 1996). Therefore several lines of evidence exist to suggest that activin B is at least produced if not functional in the human corpus luteum. In accordance with many studies (Davis *et al.*, 1987; Fraser *et al.*, 1993; Schwall *et al.*, 1990) the current study demonstrates that the βB subunit is expressed in the

primate/human corpus luteum and is indeed regulated by gonadotrophins and/or activin A (Eramaa *et al.*, 1995; Liu *et al.*, 2001; Tuuri *et al.*, 1996). However, unlike the previous reports, this study involved using the newly improved 46A/F mouse monoclonal βB antibody.

The availability of the newly developed 46A/F mouse monoclonal anti-βB, allows detection of the βB chain to be carried out with more confidence in specificity. Already the 46A/F has proven successful for use in an inhibin B ELISA such that the protocol has been simplified and quality improved (Ludlow *et al.,* personal communication) from the previous C5 antibody method (Groome *et al.*, 1996). Until recently however, detection of activin B has been met with limited success, and with no clear definite explanation (Evans and Groome, 2001). This is in contrast to the highly successful activin A assay which utilised the E4 (anti-βA) monoclonal antibody's ability to sandwich itself (Knight *et al.*, 1996). This chapter describes for the first time (and consequently still a 'work in progress') results from the 46A/F antibody for the activin B ELISA (kindly performed by Helen Ludlow) and immunohistochemistry applications.

In contrast to a previous study using an anti-rabbit βB antibody (Roberts *et al.*, 1993), the present study clearly demonstrates that the human corpus luteum expresses the βB subunit in many cell types. Staining with the 46A/F antibody localised βB to the granulosa-lutein cells and theca-lutein cells, suggesting that both steroidogenic cells types have the capacity to make activin B and potentially inhibin B (dependent upon the presence of the α chain). Activin B is indeed produced by the luteinised granulosa cells *in vitro* and concentrations from primary cultures of treated with hCG to mimic the human luteal phase mirror the expression pattern of βB. Additionally, Illingworth and co-workers have clearly shown that exogenous hCG treatments mimicking maternal recognition of pregnancy in women reduces already low inhibin B concentrations (Illingworth *et al.*, 1996), further confirming that the corpus luteum is not a source of inhibin B. There may however be a role for activin B in the corpus luteum, moreover in the late-luteal phase when the α chain and inhibin concentrations have severely declined (Fraser *et al.*, 1995; Lockwood *et al.*, 1998a; Muttukrishna *et al.*, 1995). In the late-luteal phase βB expression and subsequent inhibin B concentrations remain unchanged (Fraser *et al.*, 1995; Illingworth *et al.*, 1996). Therefore, it is tempting to speculate that during luteolysis, alike that of activin A, the human corpus luteum may be a source of activin B.

Indeed, activin A is maximal during this stage of the luteal cycle (Muttukrishna *et al.*, 1996) and has been identified a potent inducer of βB subunit expression (Eramaa *et al.*, 1995).

If activin B is up regulated or indeed an endocrine factor of the corpus luteum during the late-luteal phase, does it have the same biological actions to that of activin A? Recently, work from this thesis published evidence suggesting that activin A has a physiological role in the tissue remodelling process associated with luteolysis (Myers *et al.*, 2007a). This is indicative that hCG serves to impede activin action, to facilitate luteal maintenance by inhibiting luteolysis and allowing the maternal recognition of pregnancy (Myers *et al.*, 2007a). In order to test this hypothesis and determine if the same induction in tissue remodelling observed with activin A was applicable to activin B, primary cell cultures were treated with activin B and MMP-2 expression and activity was assessed. However, in contrast to activin A, activin B had no effect on MMP-2 in fibroblast-like cells. This suggests that activin B is not associated with the tissue remodelling during luteolysis (at least not in regards to MMP-2), however it does not rule out a potential endocrine role for activin B in the human corpus luteum. Furthermore, activin A and activin B may both be produced by the corpus luteum yet elicit differential responses. This concept could be further tested by the addition of excess activin B to determine if it can compete out activin A effects. Preliminary results however suggest that activin A and activin B added together are in fact additive (data not shown).

Clear evidence for differential developmental roles of activins is evident in mice null for βB that are viable and fertile compared to neonatal lethality seen with βA null mice (Matzuk *et al.*, 1995; Vassalli *et al.*, 1994). Such models have proven invaluable in providing an insight into the lack of functional redundancy between activin A and activin B. Indeed insertion of the inhibin βB locus into the inhibin βA locus rescues phenotypes and can partially replace activin A as an ovarian growth factor, however it is unable to maintain all aspects of follicular growth (Brown *et al.*, 2000). Furthermore, the conditional βA knockout phenotypes suggests that activin A plays an integral role in luteal remodelling as these mice are reported to have ovaries with abundant corpora lutea (Pangas *et al.*, 2007) that do not dissolute, whilst βB mice are reproductively competent. It would be interesting to compare the abundant (due to a lack tissue remodelling associated with luteolysis) corpora lutea between conditional βA null mice with those of

mice with the βB insertion into βA locus, to assess whether there is functional redundancy in regards to luteal remodelling. Such data however was not presented (Brown *et al.*, 2000).

If there are indeed differential roles of the activins, it may be a direct consequence of the receptor binding patterns elicited by each subunit. Of the three activin isoforms A, AB and B, activin B has lowest biological activity (Nakamura *et al.*, 1992), and this has often been a question indicative of the existence of different receptors for this isoform (Mathews and Vale, 1991; Nakamura *et al.*, 1992). Activins signal through type I and II serine/threonine kinase receptors and the intracellular Smads. Generally, the type I receptor that activins signal via is ALK 4, however recent data from murine pancreatic (Tsuchida *et al.*, 2004) and murine pituitary cells (Bernard *et al.*, 2006) suggest that activin B and activin AB can signal through ALK 7. Whether this is the case for the ovary or indeed the corpus luteum remains to be explored, however ALK 7 is reported to be present in granulosa cells (Wang *et al.*, 2006). In the present study although activin A and activin B had differential effects upon MMP-2 in fibroblast-like cells, they had same tendency to affect Smad 7 expression.

In conclusion, distinct patterns of activin and inhibin isoforms exist over the human menstrual cycle. This information suggests that differential roles exist not only between activin and inhibin, but also potentially between each isoform. This chapter describes the use of the newly improved βB antibody which is useful for both immunoassay and immunohistochemistry applications. Whilst the activin B ELISA is still under final development, it did produce some convincing results which mirror mRNA expression and therefore yield convincing results. Further optimisation of the activin B assay will result in more accurate individual values and development of an assay format which is applicable to both cell culture and serum samples. Consequently, concentrations of serum samples from naturally cycling women across the menstrual cycle will provide a more definitive answer regarding a potential role for activin B. Whilst this work suggests that there are potential differential effects for activin A and activin B in MMP-2 induced tissue remodelling during luteolysis, this is by no means conclusive. Further work needs to address a potential role for activin B as it is regulated differentially by hCG and activin A, suggestive that it is under paracrine and/or autocrine control in the corpus luteum. Taken together, these results suggest that activin B is a potential endocrine molecule of

the human corpus lutem that although regulated by activin A, may have a different role during luteolysis.

7 General Discussion

The corpus luteum is absolutely fundamental in the regulation of human reproduction. It is undoubtedly necessary for the maintenance of pregnancy and its demise is crucial in the initiation of the next ovarian cycle. Much of the research relating to the human corpus luteum in the current literature describes observational data which has indeed proved essential to get to our current stage of knowledge. Functional studies that do exist regarding the corpus luteum are most often in animal model systems, which although valuable regarding some aspects of luteal function, are not a good model for others. There are marked species differences in the regulation of the corpus luteum. This is most notable in the rodent corpus luteum and limits the relevance of transgenic and knockout mouse models in corpora lutea function. Indeed, even in large mono-ovular species such as ruminants the role of the uterus in luteal regulation, not seen in women, again limits the applicability of research in infra-primate species. Therefore the best model system to study the human corpus luteum is indeed, the human corpus luteum. However, it is not always practical to collect human tissue and furthermore carry out interventional procedures. This thesis reports several novel findings regarding the functional luteal lifespan with the use of an *in vivo* system of carefully dated human corpora lutea (including the novel and important stage of early pregnancy) that has been stimulated by physiological concentrations of exogenous hCG, accompanied by a more mechanistic *in vitro* model. Together these models complement each other and have proved invaluable in exploring the functional and mechanistic roles within the human corpus luteum. The complementary use of these paradigms in this thesis has allowed the development and testing of hypotheses about the paracrine regulation of human luteal function for the first time.

7.1 Gonadotrophic regulation of the human corpus luteum

It has been well recognised for many decades that gonadotrophins regulate the lifespan of the corpus luteum in women. Formation of this extraordinary endocrine gland during luteinisation is a consequence of the preovulatory LH surge, whilst maintenance of the gland during early pregnancy is absolutely dependent upon conceptus-derived hCG. Indeed, the corpus luteum is not an autonomous gland and in the absence of

gonadotrophic support its function will rapidly cease. However the mechanisms by which gonadotrophins regulate or maintain corpora lutea function remains an enigma. The corpus luteum will undergo luteolysis in the presence of maintained LH pulses (Zeleznik and Pohl, 2006). Whilst endocrinological removal of LH causes luteolysis pharmologically, the pituitary gland is not involved in regulating natural luteolysis. In ruminants $PGF_{2\alpha}$ from the uterus is the luteolysin (McCracken *et al.*, 1999). This however, is not the case in women as hysterectomy has no effect on ovarian function. These observations point to the fact that if there is a luteolysin in women it must be locally derived and locally active. That is not the case during maternal recognition of pregnancy as hCG has a clear endocrinological role in preventing luteolysis. As hCG has effects on cells that do not express the LH/hCG receptor such as fibroblasts (Duncan *et al.*, 2005b; Myers *et al.*, 2007a), immune cells (Duncan *et al.*, 1998b) and endothelial cells (Fraser and Duncan, 2005), the regulation of paracrine signalling molecules from steroidogenic cells must be involved. This suggests that important molecules involved in luteolysis or luteal rescue are LH/hCG responsive factors whose receptors are on disparate populations of luteal cells. Even now it is not clear apart from stimulating progesterone synthesis, what role hCG has upon the molecular functions, if any, in the human corpus luteum. Furthermore, in the absence of gonadotrophic support it is still uncertain how the corpus luteum undergoes luteolysis and how hCG prevents or delays this during maternal recognition of pregnancy. Previous studies leading up to this thesis, aimed to investigate these unresolved issues (Duncan, 2000; Duncan *et al.*, 2005b; Fraser *et al.*, 2005) and consequently such studies have provided the backbone to the current thesis and its investigation into corpora lutea regulation.

It is increasingly clear that hCG has the ability to inhibit luteolysis and regulate many different cells types, and their molecular functions within the corpus luteum on cells that do not necessarily express the hCG/LH receptor. One explanation for this is that hCG inhibits the production of a luteolysin from the steroidogenic cells of the corpus luteum and it is the luteolysin that is responsible for inducing genes involved in luteolysis in different luteal cell populations. Another explanation may be that hCG directly stimulates molecules that have a local luteotrophic effect that inhibit the luteolytic process that would occur in their absence. The focus of the studies presented in this thesis was to investigate paracrine signalling in the human corpus luteum. The main objectives of this thesis were (1) to identify potential paracrine molecules in the human

corpus luteum that have a luteolytic effect, (2) to identify potential paracrine molecules that have a luteotrophic effect, (3) to determine the effect of these molecules on fundamental luteal processes, (4) to establish the role that such molecules and their interactions may play in the regulation of corpora lutea function and (5) to devise a mechanistic model to explain how hCG effects disparate cell functions at a tissue level. In order to do this, combinations of human tissue and cell cultures models were utilised. The starting point was the knowledge of some regulated molecules known to be involved in luteolysis. Novel *in vivo* experiments have elegantly shown that luteal fibroblast MMP-2 production is up-regulated during luteolysis, and inhibited by hCG during maternal recognition of pregnancy in women (Duncan *et al.*, 1998a). More recently, hCG has also been shown to regulate fibroblast CTGF production in the corpus luteum (Duncan *et al.*, 2005b) and this could be modelled *in vitro* using novel cell culture methods. Using a combination of the up-regulation of MMP-2 during luteolysis, that has been shown to occur in all species investigated, and the cell culture models previously used to confirm that CTGF was regulated by hCG, through unknown paracrine intermediary molecules, activin A was identified as a possible luteolysin.

7.2 Activin A is an-anti luteal agent in the human corpus luteum

Activin A as an intermediate paracrine molecule in the human corpus luteum forms the foundation of this thesis and consequently has shaped the basis of each of the chapters presented. Synthesised predominantly by the steroidogenic cells of the human corpus luteum, activin A concentrations fluctuate over the luteal lifespan unparalleled to other hormones such as inhibin A, oestradiol and progesterone (Muttukrishna *et al.*, 1996). Many of the cell types which comprise the corpus luteum, such as the steroidogenic cells and stromal fibroblasts express the extracellular activin receptors and intracellular Smad signalling proteins required for activin signal transduction. Furthermore, the corpus luteum also possesses the ability to modulate activin action by regulation of activin inhibitors such as inhibin, follistatin and β-glycan. Activin A certainly fitted the criteria for a regulated molecule from luteal steroidogenic cells that could have actions on a wide range of cell types within the corpus luteum. More importantly however, it up-regulated the marker of luteolysis *in vitro*, MMP-2.

Peripheral serum concentrations of activin A are maximal at the time of luteolysis (Muttukrishna *et al.*, 1996). If indeed these reported serum levels reflect the intra-luteal patterns of activin A (and one would predict so, as the ovary seems to be the predominant source of regulated activin subunit expression) then it could be questioned how activin A becomes maximal during the late-luteal phase. It must be remembered that during natural luteolysis, cessation of progesterone synthesis is not due to a decline in trophic support but more appropriately abrogation of LH action at a local level. Indeed, the LH/hCG receptors do not decline in number (Duncan *et al.*, 1996a), but more likely become uncoupled from their second messenger systems which consequently result in desensitisation of the receptor (Segaloff and Ascoli, 1993). This has been thought to explain why increasing concentrations of hCG are required during pregnancy to maintain sufficient progesterone production (Duncan *et al.*, 1996a).

During the late-luteal phase, desensitised receptors, coupled with the absence of logarithmically increasing hCG serve to reduce LH action in the corpus luteum. Withdrawal of LH action will lower progesterone secretion and affect the local action of activin A. Reduced LH action will lower the inhibin α subunit leaving more chance for βA dimer formation and subsequently activin A synthesis. In addition by lowering follistatin and β-glycan expression activin action is further facilitated. This could then result in a feed-forward loop whereby activin A can continue to promote its own production, inhibit genes involved in luteal steroidogenesis (see Chapter 5) and contribute to the structural remodelling associated with luteolysis (see Chapter 3). It is likely that activin A action in the late-luteal corpus luteum is increasing rapidly and this can facilitate luteolysis. This will be self-limiting in nature as when the luteal steroidogenic cells undergo apoptosis the predominant source of activin A is removed.

7.2.1 Activin A as a luteolysin in women

A potential role for activin A during luteolysis became evident when the fibroblast cells responsible for secreting the proteolytic MMP-2 up regulated their expression and activity of MMP-2 in the presence of activin (Chapter 3). Although probably not the sole regulator of MMP-2, activin A was an attractive candidate signalling molecule for several reasons. Synthesised, secreted and transduced in the corpus luteum, activin A was clearly regulated by hCG, both directly and indirectly at the level of inhibitors. Furthermore,

peripheral serum levels were maximal during the late-luteal phase at the time of luteolysis (Muttukrishna *et al.*, 1996) and indeed when MMP-2 activity was greatest (Duncan *et al.*, 1998a). Accordingly, luteal fibroblast MMP-2 activity was up-regulated by activin A in primary cell cultures and importantly, these effects were inhibited by hCG. Through a rodent model, the importance of activin A upon structural luteolysis became further evident in the granulosa cell specific βA mouse knockout (Pangas *et al.*, 2007). These mice demonstrate an ovarian phenotype with abundant corpora lutea, providing a unique opportunity to study a role for activin A in an *in vivo* model. Collectively, these data suggest activin A plays an integral role in the tissue remodelling associated with luteolysis and that this effect might not be limited to women. It seems that one of the key roles of hCG during maternal recognition of pregnancy is to inhibit activin A action. Further work on activin A in corpora lutea mammal models is clearly indicated to replicate these findings and allow further dissection of the molecular regulation of tissue remodelling that can be further assessed in the human model used here. In order to determine if the βA conditional knockout model exhibits abundant corpora lutea due to lack of MMP-2, it would be very interesting to localise or quantify the expression of this enzyme in these mouse ovaries. Furthermore, another interesting molecule which could be assessed across the luteal phase and during maternal recognition of pregnancy and indeed in the βA null mice is α-2 macroglobulin. Studies have shown that α-2 macroglobulin inhibits both activin A and MMP-2 (Baker *et al.*, 2002; Niemuller *et al.*, 1995). Therefore it could be hypothesised that hCG may up regulate the expression of this inhibitor during maternal recognition of pregnancy to tightly regulate activin-induced tissue remodelling. Whatever the detailed mechanisms, activin A action seems to be involved in the removal of the corpus luteum (Figure 7.1).

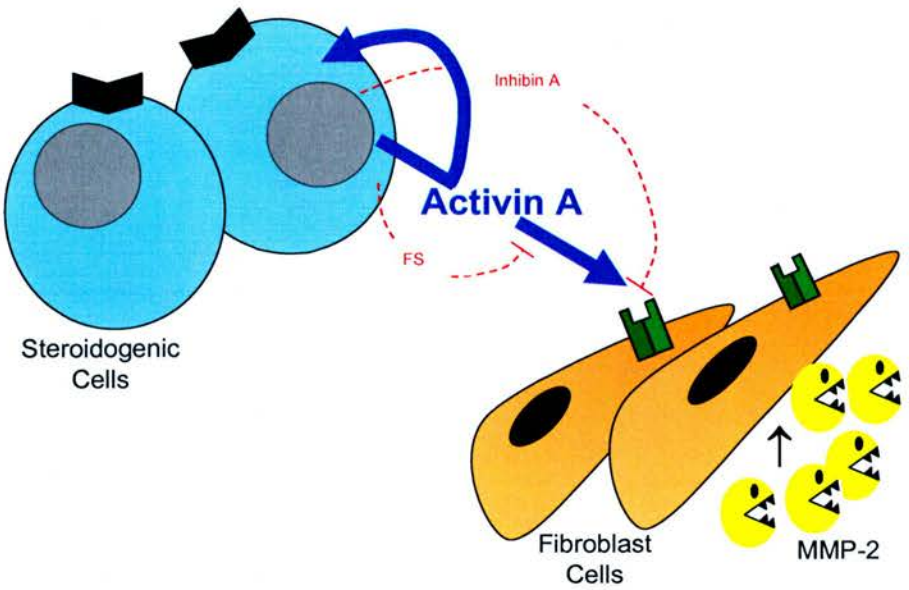


Figure 7.1 Schematic model of the proposed mechanisms which contribute to human luteolysis. In the late-luteal phase, activin A action from the steroidogenic cells is up regulated as inhibitors of activin action (inhibin A and follistatin (FS)) are markedly reduced. Fibroblast-like cells express activin receptors (green). As a result of the rising activin A levels, fibroblast-like cells up regulate the activity of MMP-2 which plays a major role in the tissue remodelling associated with luteolysis.

7.2.2 Activin A and formation of the corpus luteum

Since the studies on tissue remodelling during human luteolysis revealed that increasing activin A was important, the obvious next question is whether activin A is removed during luteal formation. As previously discussed, formation of the corpus luteum involves marked and disparate changes. The preovulatory LH surge induces the expression of important regulatory molecules that play a key role in the steroidogenesis that takes place in the newly formed granulosa-lutein cells. As discussed in Chapter 5, it is often postulated that luteinisation might be a 'default' pathway and granulosa cells are pre-programmed to undergo this transition. If this is the case, then inhibitory factors must be present within the follicular entity and are potentially products of either oocyte or granulosa cell origin. It became apparent throughout these studies that one of the roles of activin A may be to inhibit luteinisation. Whether or not activin A alone can actually prevent luteal formation or luteinisation remains elusive. However it appears to play some role in the prevention of luteal tissue. The negative effects of activin A upon key genes involved in the steroidogenic pathway, in the presence and absence of hCG suggests that one of the roles of activin A is probably to promote a more follicular type

environment, whilst hCG (mimicking the preovulatory LH surge) facilitates luteal development. Therefore it seems likely that there are similarities between the LH surge during luteal formation and hCG during luteal rescue. Both appear to, through similar mechanisms act to remove activin A action. This implies that activin A is anti-luteal at both extremes of the luteal lifespan and normal luteal function involves suppressing activin A action (Figure 7.2).

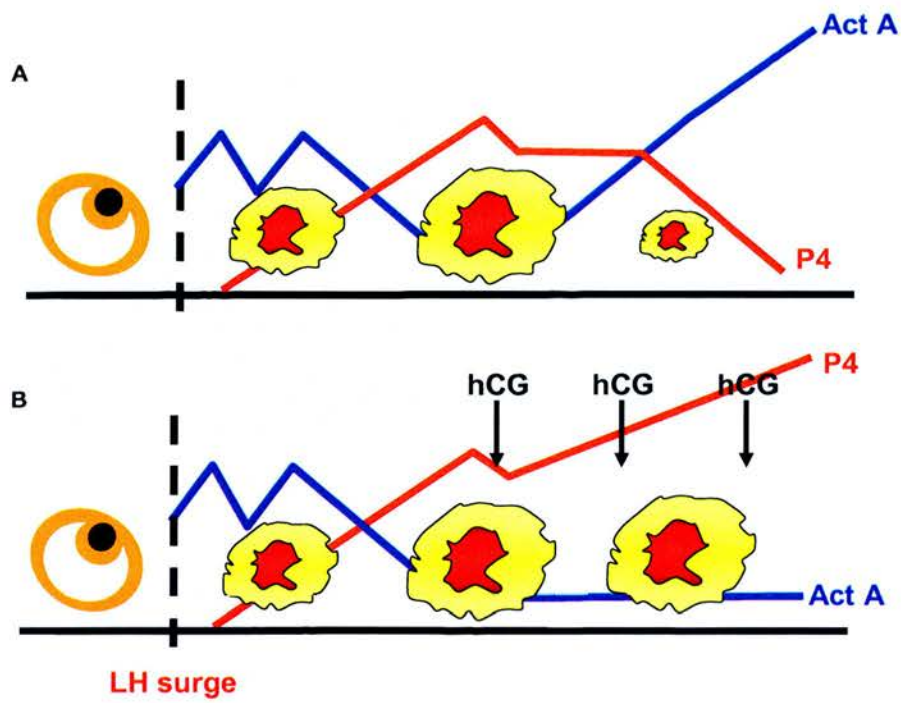


Figure 7.2 A schematic diagram representing the patterns of activin A (ActA) and progesterone (P4) secretion across the luteal phase in luteolysis and during luteal rescue with exogenous hCG. **A,** During the late-luteal phase activin A levels begin rise due to a lack of trophic support. The rise in activin A may disrupt progesterone secretion that rapidly declines at this time. Furthermore the increase of activin A also up regulates MMP-2 and tissue remodelling associated with luteolysis. **B,** In contrast however, luteal rescue is a result of hCG trophic support and consequently progesterone concentrations are maintained as it the tissue integrity as hCG suppresses activin A action within the corpus luteum.

It is unlikely that activin A alone is responsible for preventing luteinisation or promoting a follicular-like environment, however it is likely to have a major role. This has been highlighted in mouse models whereby activin A has been shown to be anti-luteal (Pangas *et al.*, 2006). Accordingly, one of the key phenotypes in mice null for the common intracellular Smad (Smad 4) gene is premature luteinisation of the granulosa cells (Pangas

et al., 2006). In parallel with Chapter 5, many of the same genes were regulated in a similar fashion as those in the Smad 4 knockout mouse. Importantly however, deletion of Smad 4 in these mice does not only interfere with activin signalling, it also interrupts TGF-β transduction. With many molecules working through the same signalling pathways it makes is hard to know whether there exists a division of labour between the activins, BMPs, and GDFs or whether they act redundantly with respect to luteinisation (Pangas *et al.*, 2006). An attractive additional candidate molecule already known to be involved in prevention of luteinisation is GDF9 (Elvin *et al.*, 1999a; Elvin *et al.*, 1999b; Eppig *et al.*, 1997; Vanderhyden *et al.*, 1993). Notably, as GDF9 uses similar Smad signalling pathways as activin it would be very interesting to see if unluteinised granulosa cells treated with activin A and GDF9 both separately and simultaneously to determine if they have similar and/or synergistic roles with respect to the prevention of luteinisation. Activin receptors are indeed localised to the oocyte in humans (Pangas *et al.*, 2002) and it could be postulated that activin therefore may signal to the oocyte to augment GDF9 expression. It should be reiterated that the granulosa cells used in the current study are already luteinised, and it remains unknown whether or not they can be 'unluteinised'. Whether or not activin A can indeed prevent or revert the luteinised phenotype of a granulosa-lutein cell remains elusive. However, there are most definitely effects at a molecular level on key genes involved in luteal formation and function which clearly antagonise or exhibit differential effects to the trophic actions of hCG.

Activin A appears to be an important paracrine molecule in the human ovary. It is anti-luteal and needs to be removed for the corpus luteum to function. One key finding of this thesis is the struggle between LH/hCG action and activin A action as both seem to be able to inhibit the effects of the other. When LH/hCG action dominates the corpus luteum retains its functional and structural integrity, however when activin A dominates, the functional and structural integrity of the tissue is lost.

7.3 A luteotrophic role for cortisol in the corpus luteum

The identification of potential luteotrophic molecules suggests that hCG many have other actions than just suppressing luteolysins. These results shed light upon the trophic effects that cortisol potentially has upon luteal maintenance. For the first time, data published from this thesis suggests that cortisol may indeed be a luteotrophic agent during maternal

recognition of pregnancy (Chapter 4; (Myers *et al.*, 2007b)). This study clearly demonstrated that the MMP-2 induced tissue remodelling associated with luteolysis was reduced in cortisol treated fibroblast-like cells. Furthermore, activin A and hCG exhibited clear differential effects upon the 11βHSD isoforms responsible for cortisol metabolism (Chapter 5) such that, by up-regulating 11βHSD1, it promoted local cortisol synthesis. It seems that during luteal rescue the suggested increase in local cortisol generation from steroidogenic cells can act on neighbouring cells with glucocorticoid receptors to inhibit MMP-2 induced tissue remodelling (Figure 7.3). Indeed, the promotion and withdrawal of local cortisol synthesis has important parallels with activin A action.

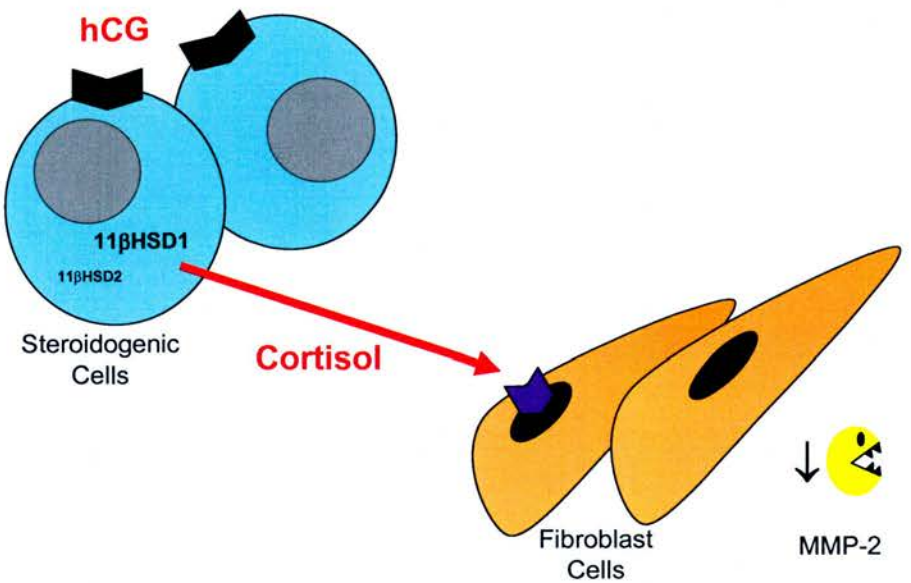


Figure 7.3 Schematic model of the proposed model of luteal rescue. During maternal recognition of pregnancy, hCG provides trophic signals to luteal cells. Steroidogenic cells, but not fibroblast cells express the LH/hCG receptor (black). HCG induces 11βHSD1 which generates cortisol in the steroidogenic cells and can act on the glucocorticoid receptor (purple) on fibroblast-like cells resulting in inhibition of MMP-2 tissue remodelling from fibroblast cells.

Activin A up regulated 11βHSD2 expression suggesting that it tends to remove cortisol from the system. Indeed activin A has been shown to inhibit cortisol secretion in other *in vitro* systems (Vanttinen *et al.*, 2003) and an important role of activin A inhibition in the corpus luteum is most likely to remove any inhibition upon MMP-2 tissue remodelling. Conversely, hCG promotes the generation of cortisol in the corpus luteum and inhibits

MMP-2 tissue remodelling associated with luteolysis (Figure 7.3). Again, this is evidence for the opposing effects of activin A and hCG on luteal cell function (Figure 7.4).

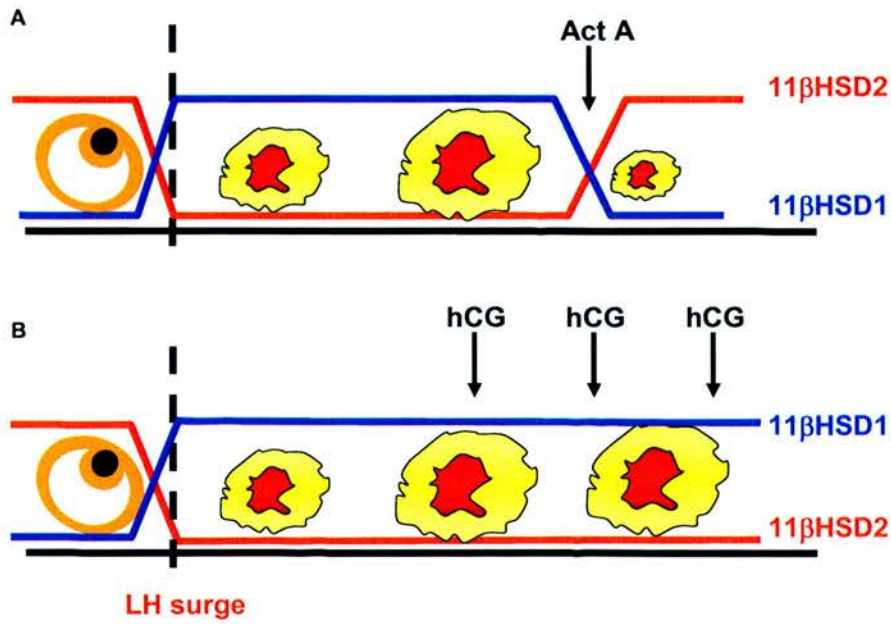


Figure 7.4 A schematic diagram depicting the differential patterns of the 11βHSD isoforms over the late follicular phase and throughout the luteal phase in luteolysis and luteal rescue with exogenous hCG. **A,** The pre-ovulatory LH surge clearly involves a switch in the predominance of 11βHSD2 to 11βHSD1, which is maintained over the luteal phase until the late-luteal phase when high activin A (ActA) levels potentially 'switch' back to a predominance of type 1 11βHSD. **B,** During luteal rescue however in the presence of gonadotrophic support, 11βHSD1 is maintained, presumably to promote generation of cortisol and inhibit the tissue remodelling associated with luteolysis.

7.4 The balance between luteotrophic and luteolytic signals

When activin A dominates in the late-luteal phase, the luteotrophic signals are inhibited. However when hCG dominates the luteolytic signals are inhibited. Although this model is simplistic and belies a large amount of complexity, it is a model system that for the first time can begin to allow us to understand the molecular regulation of the corpus luteum of women. This remains one of the great enigmas of reproductive biology. Now hypotheses can be generated and investigated using human tissue in combination with culture systems and these basic mechanistic conclusions.

In the absence of luteolysis, conceptus-derived hCG maintains the structural and functional integrity of the corpus luteum. It is likely that there are other luteotrophic and luteolytic molecules to be determined. Although the luteal rescue tissue used in the current study only mimics the first week of pregnancy, it is provides a good indicator of what might be expected until the placenta assumes principal role. Although peripheral serum levels of activin A are reported to increase over the course of pregnancy, this does not necessarily reflect intra-luteal concentrations (Muttukrishna *et al.*, 1996). Indeed the placenta has the ability to produce activins and is most likely the key source of the rising concentrations during the weeks of pregnancy. As a consequence of the work in this thesis, it is hypothesised that intra-luteal activin inhibition would continue to be maintained by hCG, at least until the luteo-placental shift so that adequate progesterone synthesis is maintained and MMP-2 tissue remodelling is limited. Furthermore there is good evidence to also suggest that active cortisol would be maintained until the luteo-placental shift as elegant rat studies demonstrate that an increase in 11βHSD type 2 is a local mechanism to facilitate luteal regression (Waddell *et al.*, 1996). Whatever happens as pregnancy progresses, the corpus luteum needs more and more trophic signals to maintain its function. This is a losing battle as the corpus luteum has no role after eight weeks gestation and the exponential rise of hCG cannot be maintained beyond 10-12 weeks. Even in the presence of hCG, the luteolysins will predominate and hCG can only delay the inevitable. This thesis suggests that a major luteolysin is activin A.

7.5 Is the role of activin B in the corpus luteum the same as activin A?

A functional role for activins within the luteal lifespan became clearly evident over the course of these studies, however it remained elusive whether these actions were unique to activin A or indeed were similar to activin B. Although a reasonable question, it has not always been particularly easy to answer. Most studies in the literature investigate activin A when considering a physiological role for activins. However, with the availability of recombinant activin B and the development of specific antibodies, more definite investigations into this protein are possible. Although still in its preliminary stages, Chapter 6 aimed to investigate a potential endocrine role of activin B in the human corpus luteum. These early results suggest that activin B has both disparate and similar effects to that of activin A. Further development of the activin B assay and assessment in serum samples from across the ovarian cycle will give a greater insight into a functional role for

this glycoprotein. The differential effects of activin B to that of activin A make it an exciting prospect to investigate, especially as it has different receptor and follistatin binding properties.

7.6 Future Work

Studies from this thesis are complete enough to stand as scientific publications in important international journals (Myers *et al.*, 2007a; Myers *et al.*, 2007b). Whilst enlightening our understanding on some of the more functional aspects of corpora lutea biology, in particular human luteolysis, the studies in this thesis have also raised many more investigative questions. There are clear pathways ahead for future studies using the work of this thesis as a starting point.

1) What is the role of activin B? Activin B effects and the relationship to hCG and activin B should be determined using similar paradigms as the investigation of activin A. This would be enhanced where antibodies or different follistatin proteins could specifically inhibit one or the other activin in the presence of the other. Already studies have shown that follistatin binds activin A at a 10-fold higher affinity than it does activin B (Schneyer *et al.*, 2003) and these studies would be of general interest as very little data is published in this field.

2) The expression of other members of the TGF-β superfamily in the human corpus luteum. The TGF-β family are markedly understudied in the human corpus luteum. Their synthesis, action and localisation is unknown. The paradigms could be used to study TGF-β, BMPs, AMH and GDF9 synthesis and regulation.

3) The interaction and actions of different TGF-β family members. As the TGF-β family members signal through the common Smad 4, it is possible that they can modulate each others actions. Pilot experiments have shown that the Smad 1,5 and 8 (involved notably in BMP signalling) are expressed in the same cells as Smad 2 and 3 (data not shown). Cell culture models should be used to dissect out these possible interactions in luteal cells.

4) Manipulation of activin A action *in vitro*. The cell culture model provides a system whereby activin A action can be manipulated. Pilot studies have shown that these cells

can be transfected wih the inhibitory Smad 7 using adenoviral vectors (data not shown). The manipulation of Smads, βA subunit, α subunit and follistatin using expression vectors and RNA interference (RNAi) technologies could be used to dissect these pathways out further.

5) The synthesis and effects of prostaglandins on luteal cells. It is likely that there are common regulators of the corpus luteum in different species. Findings from animal models may inform us of some of the common pathways to investigate. Indeed work from this thesis may inform researches working on luteolysis in non-human species including infra-primate species. Prostaglandins are major regulators in other species including the smallest primate model investigated, the marmoset monkey whereby systemic prostaglandin $F_{2\alpha}$ is luteolytic. Since last studied in women, much more information is known, and readily available, regarding prostaglandin synthesis and effects. Revisiting prostaglandins in human corpora lutea using the current molecular techniques is timely and may yield important findings.

6) Is there a change of differentiation state of granulosa lutein cells during luteolysis? Many of the molecules expressed by the steroidogenic cells during luteolysis, such as 11βHSD2, activin and possibly CTGF are expressed by follicular granulosa cells. This raises the intriguing possibility that there is a de-differentiation of luteinised granulosa cells to a more follicular or less mature phenotype during luteolysis. Preliminary evidence has shown that hCG tends to inhibit cyclin expression and promote the differentiation factor p27cip1 and that activin A does the opposite (data not shown). Indeed it was suggested at a recent international conference that luteal cells may re-enter the cell cycle as a prelude to apoptosis during luteolysis (Dr Susan Quirk, Cornell University NY, personal communication). The influence on cell differentiation in luteal cells remains uncertain and would make a fascinating story.

7.7 Conclusion

Collectively these results suggest that activin is an excellent anti-luteal molecule whose paracrine/endocrine actions are to remove or potentially inhibit luteal tissue formation, and moreover to facilitate human luteolysis. It appears that one of the physiological roles of the gonadotrophins in the human corpus luteum is to impede activin action. The

preovulatory LH may serve to promote luteinisation by subsequently inducing expression of key genes that are involved in successful luteal formation, and equally as important, suppress activin A which may be detrimental to the system. Additionally, during maternal recognition of pregnancy, the biological actions of activin A remain inhibited by conceptus-derived hCG that has marked and disparate changes on surrounding cell types that do not expression the hCG receptor. In addition to suppressing activin A-induced proteolytic activity, hCG will facilitate cortisol production via up-regulation of 11βHSD1, to further impede MMP-2 tissue remodelling. Consequently, luteolysis is prevented, as luteal fibroblast-like MMP-2 is inhibited and hCG-derived cortisol production promotes the maternal recognition of pregnancy. In summary this thesis for the first time identifies activin A as an excellent candidate molecule for the elusive luteolysin in women, and highlights cortisol as a potential novel luteotrophic molecule.

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In Vitro Evidence Suggests Activin-A May Promote Tissue Remodeling Associated with Human Luteolysis

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Luteolysis in women is associated with an up-regulation of the expression and activity of matrix metalloproteinase-2 (MMP-2), which is inhibited by human chorionic gonadotropin (hCG) during maternal recognition of pregnancy. Because the primary source of MMP-2 is fibroblasts that do not express LH/hCG receptors, we aimed to investigate the regulation of MMP-2. Women with regular cycles having hysterectomy for nonmalignant conditions and women undergoing oocyte retrieval for assisted conception were used in this current study. Novel primary cultures and cocultures of luteinized granulosa cells and fibroblast-like cells in conjunction with human corpora lutea from different stages of the luteal phase were used to investigate the role of activin-A in the corpus luteum. The effect of hCG, activin-A, and follistatin on MMP-2 activity and expression was assessed by gelatin zymography and quantitative RT-PCR in primary cell cultures. Confirmation of signaling pathways involved in the activation of MMP-2 was assessed by immunofluorescence, RT-PCR, and quantitative RT-PCR. In primary cell culture, steroidogenic cells secrete activin-A and its inhibitors, inhibin-A and follistatin. Follistatin expression is up-regulated by hCG ($P < 0.05$). The fibroblast-like cells producing MMP-2 have the machinery for activin reception, expressing both type I and type II activin receptors and Smad proteins. Activin-A up-regulated both activity and expression of MMP-2 in fibroblast-like cells ($P < 0.05$). This activity was inhibited in cocultures of luteinized granulosa cells and fibroblast-like cells in the presence of hCG ($P < 0.05$) or follistatin ($P < 0.01$). Activin-A is an excellent candidate for an effector molecule in human luteolysis whose paracrine action is inhibited during maternal recognition of pregnancy. (*Endocrinology* 148: 3730–3739, 2007)

THE HUMAN CORPUS luteum is a highly vascular and active endocrine gland that in the midluteal phase measures up to 2 cm in diameter. However, unless human chorionic gonadotropin (hCG) is secreted by a conceptus, this highly transient structure will undergo functional and structural luteolysis (1), becoming a small fibrous remnant within a matter of days. The process of luteolysis is associated with marked tissue remodeling and vascular involution. Cellular processes that are known to occur during the regression of the human corpus luteum include cell death (2), an increase in the expression of connective tissue growth factor (3), an influx of macrophages (4), and an up-regulation of fibroblast matrix metalloproteinase-2 (MMP-2) expression and activity (5).

MMPs are key proteolytic enzymes involved in the degradation of the extracellular matrix, which is constantly remodeled during luteolysis. Much evidence exists to suggest that MMP-2 is an important luteolytic agent during the demise of the corpus luteum. In rats, structural luteolysis was associated with an increase in MMP-2 activity (6), whereas in pigs, the expression of MMP-2 was elevated in the regressing corpus luteum (7). Additionally, primates (8) and humans (5) show maximal MMP-2 expression in the late luteal phase, positively correlating with the functional and structural regression of the corpus luteum. Furthermore, hCG during maternal recognition of pregnancy in women (5) reduces the expression of MMP-2. Because MMP-2 is up-regulated during luteolysis, and conversely inhibited in the presence of hCG during maternal recognition of pregnancy, it is likely that it has an important role in the luteolytic process.

The primary source of MMP-2 in the human corpus luteum is the luteal fibroblasts that do not express LH/hCG receptors (5), suggesting that there is a paracrine regulator of MMP-2 expression (9). We therefore hypothesized that in the corpus luteum, MMP-2 is regulated by hCG through an intermediate molecule. Unlike LH, FSH, progesterone, estradiol, or inhibin-A, the concentrations of activin-A in serum are maximal during the late luteal phase (10), suggesting it may have a positive luteolytic action. Activins and other members of the TGF-β superfamily are known to control many diverse physiological processes (11); therefore, we hypothesized that activin-A may be a critical intermediate signaling molecule in the human corpus luteum.

As described previously (4, 5, 12), we have developed a system for the collection of carefully dated human corpora lutea and more recently (3) a primary cell culture system using human luteinized granulosa cells and/or novel cultures of fibroblasts-like cells derived from the luteinizing follicle. The aim of the current study was to investigate whether activin-A was a paracrine regulator of luteal tissue remodeling by investigating its effects on MMP-2 expression and activity using our model systems.

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Abbreviations: ActRIIA, Activin receptor IIA; ALK4, activin receptor like-kinase 4; G6PDH, glucose-6-phosphate dehydrogenase; hCG, human chorionic gonadotropin; MMP-2, matrix metalloproteinase-2; NGS, normal goat serum; TBS, Tris-buffered saline.

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Materials and Methods

Collection of human corpora lutea

Tissue collection was approved by the local medical research ethics committee, and all women gave informed consent. Human corpora lutea (n = 18) collected over the past 12 yr (3, 5) were enucleated at the time of surgery from women with regular menstrual cycles undergoing hysterectomy for benign conditions and dated on the basis of the urinary LH surge as described previously (9, 13). In this study, six corpora lutea were classified as early-luteal (LH+1 to LH+5), six as mid-luteal (LH+6 to LH+10), and six as late-luteal (LH+11 to LH+14). At operation, the corpus luteum was quartered to ensure that each quarter contained all cellular elements. Two quarters were immediately frozen and stored at −70 C until RNA extraction was carried out. The remaining tissue was fixed in 10% neutral buffered formalin for subsequent immunohistochemistry.

Isolation of human luteinized granulosa cells and derivation of fibroblast-like cells

The medical ethics committee separately approved the collection of cells from patients undergoing assisted conception. With patient consent, follicular fluid was collected from women undergoing transvaginal oocyte retrieval for *in vitro* fertilization after ovarian stimulation using a standard procedure (14). Isolation of luteinized granulosa cells using Percoll gradient centrifugation was carried out as described previously (3). Fibroblast-like cells were obtained from prolonged cultures of follicular aspirates as described previously (3).

Derivation of fibroblast-like cells from human corpora lutea

Corpora lutea collected during surgery (as described above) were minced in cell culture conditions and placed in flasks containing 10% fetal bovine serum as described previously (3). Cultures were left to reach confluence and grown until sufficient numbers of cells (60,000 per well) could be obtained for experimental procedures described below.

Primary cell culture

Fibroblast-like cells (60,000 per well) were added to 24-well plates. After 24 h in serum-free conditions (supplemented DMEM/F12 Ham mixture; Life Technologies, Inc., Gaithersburg, MD), as described previously (3), fresh medium was added containing human recombinant activin-A (R&D Systems, Inc., Abingdon, UK; 10–100 ng/ml and controls) or inhibin-A (NIBSC, Hertfordshire, UK; 25 ng/ml and control). All controls contained the carrier solution equivalent to the highest concentration added. After 24 h, culture medium was stored for subsequent analysis, and cells were collected for mRNA extraction. Three to four replicates were performed in at least three separate experiments.

Pooled luteinized granulosa cells (100,000 per well) were cultured in 24-well plates precoated with Matrigel (BD Biosciences, Bedford, MA) in serum-free medium (supplemented DMEM/F12 Ham mixture), as described previously (3). Medium was changed every 2–3 d over the course of the culture period. After 6–8 d in culture, a maximally stimulating dose of hCG (Serono Laboratories, Welwyn Garden City, UK; 100 ng/ml and control) was added. After 24 h, medium and cells were stored for subsequent analysis and mRNA extraction. Three experimental replicates were carried out in duplicate.

For coculture experiments, approximately 60,000 fibroblast-like cells were added to the wells containing approximately 100,000 luteinized granulosa cells after culture for 5–7 d. After coculture for 24 h in serum-free medium, fresh serum-free medium was added containing either hCG (100 ng/ml and control) or follistatin (R&D Systems; 500 ng/ml and control). Follistatin treatments were within physiological levels (100–600 ng/ml) of that previously reported in human follicular fluid (15). Each control contained the carrier solution equivalent to the highest concentration added. After 24 h, mRNA was extracted from cocultures and controls consisting of fibroblasts only. Each coculture and control treatment was replicated six times in three separate experiments.

Preparation of cDNA from corpora lutea and cultured cells and primer design

mRNA was batch extracted from both frozen human corpora lutea and cultured cells and reverse transcribed into cDNA using random hexamers as described previously (3). Oligonucleotide PCR primers for each gene investigated were designed using Primer3 software (http://primer3.sourceforge.net/) from DNA sequences obtained from GenBank (www.ncbi.nlm.nih.gov). Primers were synthesized by MWG-AG Biotech (Milton Keynes, UK) and the 5′ to 3′ sequences used in this study are listed in Table 1. PCR conditions were as follows: incubation at 95 C for 5 min, 35 cycles of 95 C for 30 sec, appropriate annealing temperature (Table 1) for 30 sec, and 72 C for 90 sec, followed by 72 C for 10 min. PCR products were separated by applying 110 V for 1 h to 2% agarose gels with ethidium bromide and visualized and photographed under UV transillumination.

Quantitative analysis of gene expression by RT-PCR

Each assay was optimized using PCR amplification of human placental cDNA. The assays were optimized for $MgCl_2$ concentrations and annealing temperatures. PCR amplifications were performed using Thermostart *Taq* (AB Gene, Surrey, UK) in a DNA Engine gradient cycler (MJ Research, Inc., Watertown, MA) as previously described (3). Data were normalized according to the expression level of glucose-6-phosphate dehydrogenase (G6PDH), determined in duplicate by reference to a serial dilution calibration curve generated for each sample using the standard LightCycler software.

Gelatin zymography

Cell culture medium was collected from serum-free cultures and subsequently frozen at −20 C. Aliquots of 200 μl were subjected to freeze drying for 2–3 h until they resembled a powder and then reconstituted in 20 μl sterile dH_2O. One microliter of the reconstituted sample was added to sample buffer [10% (vol/vol) glycerol, 1% (wt/vol) SDS, and 0.04% (vol/vol) bromophenol blue] and applied to an 11% (wt/vol) polyacrylamide gel containing 1 mg/ml gelatin and 0.1% (wt/vol) SDS. Gels were incubated in 2.5% Triton X-100 for 45 min after electrophoretic separation of proteins and then incubated at 37 C overnight in digestion buffer [50 mmol/liter Tris-HCl (pH 7.6) containing 0.2 mol/liter NaCl, 5 mmol/liter $CaCl_2$, and 0.02% (wt/vol) Brij 35] as described previously

TABLE 1. Sequence of primers and annealing temperatures used for qualitative and quantitative RT-PCR

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Annealing temperature (C)
MMP-2	ATGACAGCTGCACCACTGAG	ATTTGTTGCCCAGGAAAGTG	61
G6PDH	CGGAAACGGTCGTACACTTC	CCGACTGATGGAAGGCATC	62
Inhibin α	CCAGCTGTGAGGACAAGTCA	CTAGCAGGGGCTCAGAGCTA	56
βA	AGACGCTGCACTTCGAGATT	CCCTTTAAGCCCACTTCCTC	56
Follistatin	CAGTAAGTCGGATGAGCCTGTCT	CCTGGTCTTCATCTTCCTCCTCCT	69
β−Glycan	CTGTTCACCCGACCTGAAAT	CGTCAGGAGGCACACACTTA	67
ActRIIA	TTTCCGGAGATGGAAGTCAC	GTCCTGGGTCTTGAGTTGGA	54
Alk2	AAGGCAGGTATGGTGAGGTG	ACCACAGCTGGGTACTGGAG	55
Alk4	GAGATTGTGGGCACTCAAGGG	AGCTGGGACAGAGAGTCTTCTTG	60
Smad2	CTGGGATGGAAGAAGTCAGC	CAGTCCCCAAATTTCAGAGC	55
Smad3	TGAGGCTGTCTACCAGTTGACC	CCGCTGTTCCAGTGTGTCTTAG	57
Smad4	GATTGCAGACCCACAACCTT	CTAGGAGCAAGGCAGCAAAC	54

(5). Gels were stained in staining solution [30% (vol/vol) methanol, 10% glacial acetic acid, and 0.5% (wt/vol) Coomassie brilliant blue G250] and then destained in the same solution minus the Coomassie staining dye. The bands on the zymography gels reflect the activity of MMP-2 and were analyzed by transmission densitometry (G-700 densitometer; Bio-Rad, Hemel Hempstead, Hertsfordshire, UK) and integrated software (Quantity One; Bio-Rad). All densitometry measurements were made between samples on the same gel or between gels run under identical conditions with a common control sample on each gel to ensure comparability.

Measurement of inhibin-A and activin-A

Inhibin concentrations in culture medium collected from luteinized granulosa cells were measured using a plate modification of a standard in-house inhibin-A RIA. The sensitivity of this assay was 2 pg/ml and was carried out as previously described (16). Activin-A concentrations were measured using a two-site ELISA kit that measured total activin-A (17) (Oxford Bio-Innovation, Oxfordshire, UK), following the manufacturer's instructions. This assay had a sensitivity of less than 78 pg/ml and inter- and intraplate coefficients of variation of less than 10%.

Immunohistochemistry

Immunolocalization was carried out using antibodies recognizing phosphorylated Smad 2/3 (New England Biolabs, Hertfordshire, UK), activin βA subunit (concentration 4.3 μg/ml, kindly provided by Prof. N. Groome, Oxford Brookes University, Oxford, UK), activin receptor IIA (ActRIIA), and activin receptor like-kinase 4 (ALK4) (kindly provided by R. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden) in 5-μm paraffin tissue sections of human corpora lutea prepared on poly-L-lysine-coated microscope slides. These sections were dewaxed, rehydrated, and washed in Tris-buffered saline (TBS)/0.1% Tween 20 and TBS, respectively. Sections for phosphorylated Smad 2/3 were subjected to microwave antigen retrieval in 0.01 M citric acid (pH 6.0) for 10 min and left to cool to room temperature. All sections were washed and placed in 3% H_2O_2/methanol for 30 min to block any endogenous peroxidase activity.

Normal goat serum (NGS; Diagnostics Scotland, Edinburgh, UK) diluted 1:4 in TBS containing 5% BSA (NGS/TBS/BSA) was added to phosphorylated Smad 2/3, ActRIIA, and ALK4 sections, whereas βA sections were blocked in normal rabbit serum (Diagnostics Scotland) 1:5 in TBS for 1 h at room temperature. Primary antibodies were diluted in respective blocking solutions and incubated on sections overnight at 4 C (phosphorylated Smad 2/3, 1:1000; βA, 1:500; ActRIIA, 1:400; ALK4, 1:400). Sections were rinsed twice for 5 min each time in wash buffers and incubated with secondary antibodies [for phosphorylated Smad 2/3, ActRIIA, and ALK4, biotinylated goat-antirabbit IgG diluted 1:500 in NGS/TBS/BSA; for βA, rabbit-antimouse diluted 1:25 in TBS (Dako Corp., Cambridge, UK)].

Incubations lasted for 1 h and were followed by two washes for 5 min. Thereafter, phosphorylated Smad 2/3, ActRIIA, and ALK4 sections were incubated in avidin-biotin complex-horseradish peroxidase (Dako) for 1 h, and βA sections were incubated in mouse peroxidase-antiperoxidase (Dako) diluted 1:100 in PBS. Incubations were at room temperature for 1 h, and all sections were washed in TBS (twice for 5 min each) and bound antibodies visualized by incubation with liquid 3,3′-diaminobenzidine tetrahydrochloride (Dako). Sections were counterstained lightly with hematoxylin to enable cell identification. Negative controls for each antibody examined were performed identically to the above protocol with primary antibody incubations substituted with blocking serum containing nonspecific Igs at the same concentration. Images were captured using an Olympus Corp. Provis microscope (Olympus Corp. Optical Co., London, UK) equipped with a Kodak DCS330 camera (Eastman Kodak Co., Rochester, NY), stored on an HP computer and assembled using Photoshop 7.0.1 (Adobe Systems Inc., Mountain View, CA).

Double-fluorescent immunohistochemistry

Sections were washed, subjected to antigen retrieval, and blocked as described above. Negative controls were performed as above. Washes detailed below were for 5 min each, and incubations were at room temperature unless otherwise specified. Rabbit anti-phosphorylated Smad 2/3 was diluted 1:100 in NGS/TBS/BSA (see above) and incubated on sections overnight at 4 C. Sections were washed, incubated with goat antirabbit IgG 488 (Dako) diluted 1:200 in PBS for 1 h, and then washed in PBS. Sections were reblocked with NGS/TBS/BSA for 1 h and then incubated with mouse anti-CD31 or mouse anti-CD68 (Dako) diluted 1:20 in NGS/TBS/BSA overnight at 4 C. Sections were washed, incubated with biotinylated goat antimouse IgG (Dako) diluted 1 in 500 in NGS/TBS/BSA for 30 min, and then washed in TBS.

The fluorochrome streptavidin 546 Alexafluor (Molecular Probes, Inc., Eugene, OR) diluted 1:200 in PBS was incubated on slides for 1 h. For the labeling of nucleic acids, sections were counterstained with a nuclear-specific blue fluorescent label (To-Pro 3; Molecular Probes) diluted 1:2000 in PBS for 2 min. Sections were washed and mounted in Permafluor (Beckman Coulter, High Wycombe, UK). Fluorescent images were captured using an LSM 510 Axiovert 100M confocal microscope (Carl Zeiss Ltd., Welwyn Garden City, UK). Images were compiled using Photoshop 7.0.1 (Adobe Systems).

Statistical analysis

Statistical analyses were carried out, after confirmation of normal distributions for parametric analysis, using a paired *t* test when treatment and control samples were analyzed or with ANOVA when more than two treatments were analyzed. Where significant differences were observed ($P < 0.05$) using ANOVA, pairwise comparisons were carried out using Bonferroni's multiple comparisons test. All statistical tests are highlighted in the figure legends, and differences are given as either *, $P < 0.05$; **, $P < 0.01$; or ***, $P < 0.001$. Differences were considered significant at $P < 0.05$.

Results

MMP-2 and MMP-9 activity in primary cell culture

MMP activity was examined in primary cell cultures of luteinized granulosa cells and fibroblast-like cells using gelatin zymography (Fig. 1A). Primary cultures of luteinized granulosa cells secreted MMP-9, whereas little MMP-2 activity was detected. Conversely, and as anticipated, fibroblast-like cells secreted large amounts of MMP-2. Cocultures of luteinized granulosa cells and fibroblast-like cells secreted both zymogens.

The effect of activin-A on the expression of MMP-2 in fibroblast-like cells

MMP-2 activity in primary cell cultures was increased by activin-A in a dose-dependent manner (10–100 ng/ml; $P < 0.05$; $r^2 = 0.55$, by linear regression). Because intrafollicular concentrations of activin-A have been reported to be in the range of 0.42–26.3 ng/ml (18), we investigated the response to physiological concentrations of activin-A (10 and 25 ng/ml) in detail (Fig. 1B). Both MMP-2 expression ($P < 0.05$, by ANOVA) and activity ($P < 0.05$, by ANOVA) were increased when compared with controls when fibroblast-like cells were exposed to 25 ng/ml for 24 h (Fig. 1B). Correspondingly, the primary cultures of fibroblasts expressed activin receptors (I and II) and Smad 2, 3, and 4 as well as MMP-2 (Fig. 1D). We believe that the effect of activin-A on fibroblast-like cells is specific because cells exposed to inhibin-A show no change in MMP-2 production ($P > 0.05$, paired *t* test, data not shown). Furthermore, pilot studies (n = 2 corpora lutea) on fibroblast-like cells obtained from prolonged cultures of disaggregated human corpora lutea exposed to 25 ng/ml activin-A also demonstrated the same effects on MMP-2 expression (Fig. 1C). It is therefore likely that activin-A could

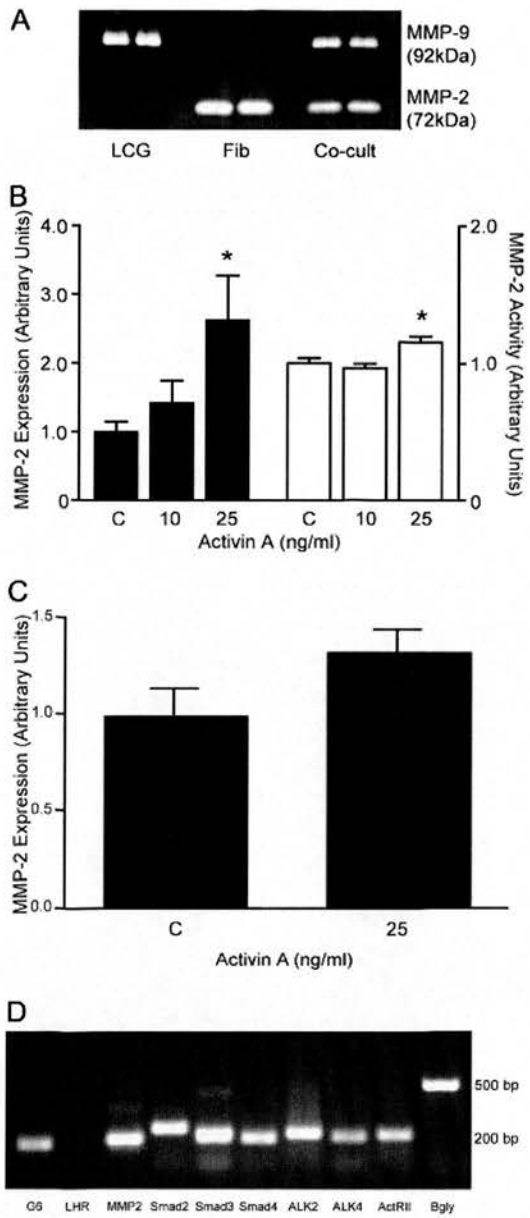

FIG. 1. MMP-2 activity and expression in primary cell cultures. A, Representative gelatin zymogram of primary and cocultures of luteinized granulosa cells and fibroblast-like cells. Luteinized granulosa cells (LGC) secreted MMP-9 and very little MMP-2. Fibroblast-like cells (Fib) secreted only MMP-2 whereas cocultures (Co-cult) of the two cell types show activity for both zymogens. B, Activin-A increases MMP-2 in primary cultures of fibroblast-like cells. Relative MMP-2 mRNA expression, using real-time quantitative RT-PCR, is shown in *black bars*, and MMP-2 activity, using gelatin zymography, is shown in *white bars*. MMP-2 expression ($P < 0.05$, by ANOVA) and activity ($P < 0.05$, by ANOVA) was significantly increased in fibroblast-like cells exposed to 25 ng/ml activin-A in culture (n = 3–4 experiments). C, Pilot studies using fibroblast-like cells derived from disaggregating human corpora lutea (n = 2) demonstrate the same effect for MMP-2 expression to increase in the presence of 25 ng/ml of activin-A. D, Fibroblast-like cells show mRNA expression for housekeeping gene G6PDH (G6) and MMP-2; however, they do not express the LH receptor (LHR), indicative that the activin action via hCG mechanisms is under paracrine control. Furthermore, the expression of activin receptors and signaling pathways can be confirmed in these cells. Bands represent 200 and 500 bp as indicated. Bgly, β-Glycan.

act as a regulator of luteal fibroblast MMP-2 production at physiological concentrations.

Identification of activin synthesis and action in human corpora lutea

Corpora lutea, at each stage of the luteal phase, have the potential to synthesize activin-A, respond to activin-A, and inhibit its action because they express mRNA for βA subunit, activin receptors (I and II), Smad 2 and 3, and the common Smad 4 as well as inhibin α-subunit, β-glycan, and follistatin. The activin βA subunit is localized to the LH-responsive steroidogenic cells of corpora lutea at each stage of the luteal phase (Fig. 2A). Although activin receptors can be found on luteal steroidogenic cells, it is notable that luteal fibroblasts, which secrete MMP-2, express both activin receptors I and II (Fig. 2, B and C) and nuclear phosphorylated Smad 2/3 (Fig. 2D) at each stage of the luteal phase.

Change in Smad signaling across the luteal phase

We investigated phosphorylated Smad 2/3 across the luteal phase by immunohistochemistry (Fig. 2, D–H). Nuclear phosphorylated Smad 2/3 could be detected at all stages of the luteal phase in both the steroidogenic (granulosa lutein cells and theca lutein cells; Fig. 2D) and stromal cell compartments. Although it was detected in the fibroblast layer, it was clear that not all cells in this layer immunostained equally. We therefore colocalized phosphorylated Smad 2/3 with other cell markers. Very little phosphorylated Smad 2/3 immunostaining was noted in endothelial cells (CD31-positive cells) when compared with neighboring stromal cells (Fig. 2E) at any stage of the luteal phase, although low-level staining could be noted in some cells of vessels (Fig. 2F). In all sections examined, the fibroblast layer contained macrophages (CD68-positive cells). Dual immunostaining suggested that there was less phospho-Smad 2/3 immunostaining in the macrophages (Fig. 2, G and H) than the fibroblasts.

To investigate changes in Smad signaling across the luteal phase, the expression of Smad 2 and Smad 3, in carefully dated corpora lutea, was investigated using quantitative RT-PCR (Fig. 3). Smad 3 expression increased to a maximum in the late luteal phase (Fig. 3B) and was different between early and mid ($P < 0.05$, by ANOVA) and early and late ($P < 0.01$, by ANOVA) (Fig. 3B). The expression of Smad 2 mRNA (Fig. 3A) was also assessed over the luteal phase, and although it demonstrated a similar trend to that of Smad 3, the differences did not reach significance.

Effect of hCG on activin-A and its inhibitors in primary cell cultures of steroidogenic cells

To test our hypothesis that during maternal recognition of pregnancy, the exposure of hCG to the corpus luteum results in less activin-A signaling from the steroidogenic cells, we assessed the effect of hCG in primary cultures of luteinized granulosa cells. The secretion of both inhibin-A (range, 10–500 pg/ml; $P < 0.05$, by t test) and activin-A (range, 1–5 ng/ml; $P < 0.05$ by t test) was increased by the addition of hCG within 24 h (Fig. 4, A and B). Experimental results were

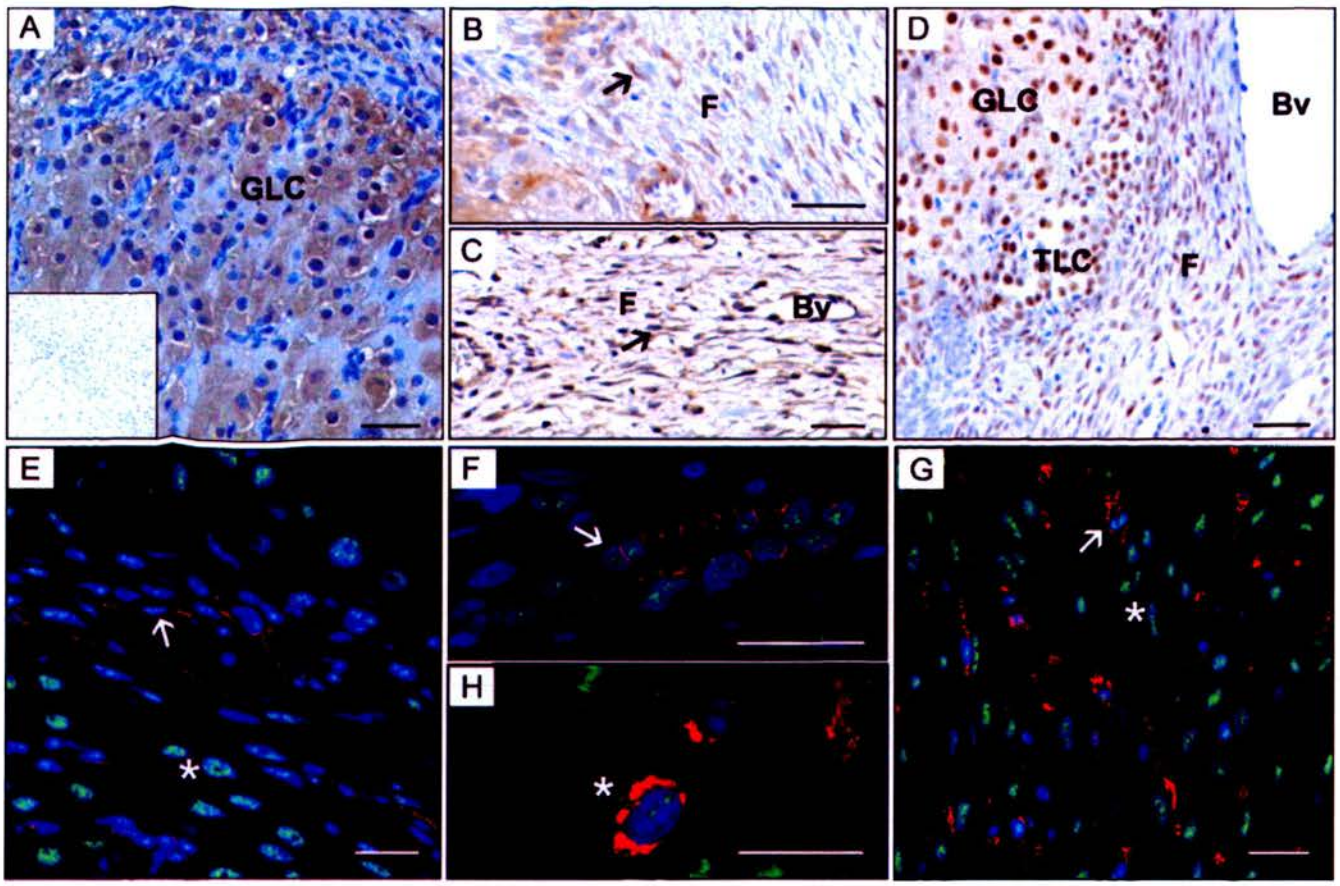

FIG. 2. Immunolocalization of factors involved in activin/TGF-β signaling in human corpora lutea. A, Light-field microscopy of a late-luteal human corpus luteum showing positive brown staining of the βA-subunit in the granulosa-lutein cells (GLC) with little or none observed in the surrounding fibroblast layer. *Inset* shows no staining in the negative control serial section. B and C, Positive staining for the activin receptors ALK4 (B) and ActRIIA (C) in the surrounding fibroblast layer of a mid-luteal corpus luteum localized to cells that resemble fibroblast cells (*arrows*). D, Phosphorylated Smad 2/3 is expressed during all stages of the luteal phase. Positive staining for this receptor-activated Smad is evident in the nucleus of granulosa-lutein cells (GLC), thecal-lutein cells (TLC), and surrounding fibroblast layer (F) of the corpus luteum with little staining around blood vessels (Bv). E, Double immunofluorescence of endothelial cells (CD 31) (*arrow*) in *red* colocalized with phosphorylated Smad 2/3 (*green*) in a mid-luteal corpus luteum. Nuclear phosphorylated Smad staining is clear in presumptive fibroblast cells (*asterisk*) located in the stromal area of the corpus luteum and less marked in the endothelial cells (*arrow*). Nuclear staining is depicted in *blue*. F, High-power images illustrate a low level of phosphorylated Smad 2/3 (*green*) in some endothelial cells (*arrow*) in *red*. G, Low-power image of phosphorylated Smad 2/3 (*green*) and macrophages (CD 68-positive cells) (*red*) in a late-luteal corpus luteum. Phosphorylated Smad 2/3 is localized to fibroblast cells (*asterisk*) and less so in the macrophages (*arrow*) stained in *red*. H, Higher-power view of an individual macrophage (*red*) in the same tissue section (*asterisk*) showing low levels of phospho-Smad (*green*). *Scale bars*, 40 μm (A–D) and 20 μm (E–H).

standardized to their controls to consider variation within individual experiments. Although exposure to hCG did not change the inhibin:activin ratio, luteinized granulosa cells markedly increased their expression of the activin-binding protein follistatin ($P < 0.01$, by t test) in response to hCG stimulation (Fig. 4C). In the presence of hCG, the up-regulation of follistatin may therefore be able to reduce the local actions of activin-A.

Evidence that luteal MMP-2 is under the paracrine control of activin-A

To test our hypothesis that hCG could inhibit MMP-2 expression in a paracrine signaling fashion, we employed a novel primary cell coculture system of luteinized granulosa cells and fibroblast-like cells (3). In these cocultures, the primary source of MMP-2 in the culture medium are the fibroblast-like cells (Fig. 1A). The addition of hCG to fibroblast-like cell cultures had no effect on the expression of MMP-2 ($P > 0.05$, t test; Fig. 5A). However, cocultures exposed to hCG demonstrated a significant reduction in the expression of MMP-2 ($P < 0.05$, by t test; Fig. 5A), suggestive that hCG is acting on luteinized granulosa cells to influence fibroblast-like cell MMP-2 expression in a paracrine manner.

To test our hypothesis that hCG is manipulating MMP-2 expression through a reduction in activin-A activity secondary to an up-regulation of follistatin expression, we added physiological concentrations of follistatin to our primary cell cultures. The addition of follistatin to cultures of fibroblast-like cells had no significant effect upon MMP-2 expression ($P > 0.05$, t test; Fig. 5B). However, follistatin had the same effect as hCG in reducing the expression of MMP-2 in co-

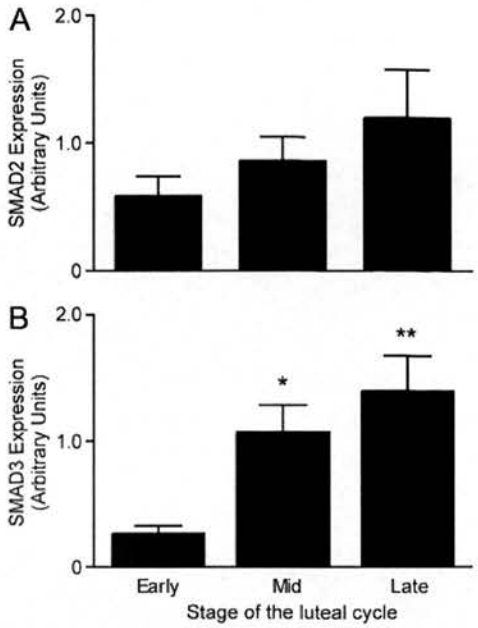

FIG. 3. Expression of the receptor-activated Smads in human corpora lutea over the luteal cycle. A, Real-time quantitative RT-PCR demonstrated that the mRNA expression of Smad 2 increased over the luteal phase (n = 6 for each group); however, a nonsignificant trend ($P > 0.05$, by ANOVA) was observed. B, The expression of Smad 3 was significantly increased from the early luteal phase in both mid-luteal ($P < 0.05$, by ANOVA) and late-luteal ($P < 0.01$, by ANOVA) samples.

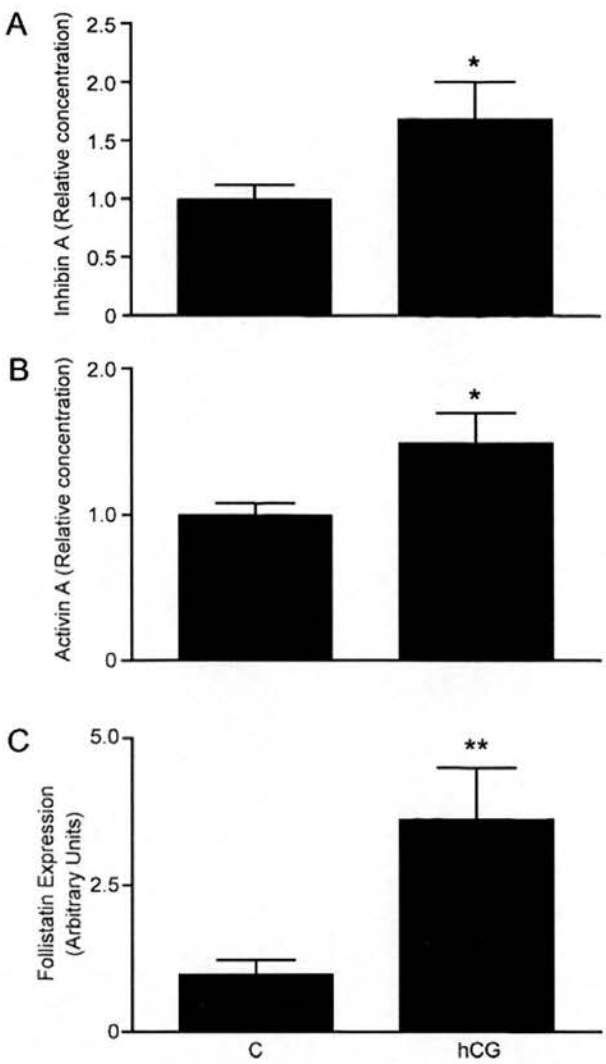

FIG. 4. The effect of hCG on elements of the activin pathway in primary cell cultures (n = 3). A and B, Luteinized granulosa cells show an increase in inhibin-A (A) ($P < 0.05$, by *t* test) and activin-A (B) ($P < 0.05$, by *t test*) production when treated with hCG (100 ng/ml) for 24 h relative to control samples. C, The expression of the activin binding protein follistatin ($P < 0.01$, by *t* test) is up-regulated in the presence of hCG (100 ng/ml); n = 3 separate experiments.

cultures of luteinized granulosa cells and fibroblast-like cells (Fig. 5B; $P < 0.01$, by *t* test).

Discussion

How luteolysis occurs in women and how it is inhibited by hCG during maternal recognition of pregnancy is not yet understood. What is clear, however, is that the inhibition of luteolysis by hCG involves disparate effects on cell types in the corpus luteum that do not express LH/hCG receptors. We have previously shown that hCG regulates luteal fibroblast (3, 5), macrophage (4), and endothelial cell (19) function. This means that paracrine signaling molecules from steroidogenic cells have key roles in the local regulation of luteal cell function. To date, the identity of these molecules has remained largely elusive. Here we have shown for the first time, using a combination of observational studies on human corpora lutea combined with interventional studies using human primary cell culture and coculture models, that activin-A is an excellent candidate molecule for a paracrine regulator of luteal remodeling during luteolysis whose action can be inhibited by hCG during maternal recognition of pregnancy.

It has to be highlighted that these studies used luteinized granulosa cells and fibroblast-like cells from the luteinizing follicle. We are using this coculture model to inform us about the paracrine interactions that occur during luteolysis. It is possible that dispersed cells from human corpora lutea may respond differently at different stages of the luteal phase. Unfortunately, such comprehensive studies using fresh human luteal tissue are almost impossible, and it is likely that analysis of activin effects on remodeling of luteal cells will need further assessment in subhuman species.

Activins belong to the structurally related TGF-β superfamily that includes inhibins and bone morphogenetic proteins. Members of this family have been shown to have important paracrine regulatory roles in diverse physiological processes (11). Indeed, activin signaling has been shown to be essential in inflammation (20), cell proliferation and apoptosis (21), fetal development (22), and male reproduction (23, 24). In particular, it has been established that activins may have a paracrine role during the normal ovarian cycle (24, 25). Activin can stimulate the proliferation of granulosa cells in small follicles (26) and enhance their expression of FSH receptors and aromatase (24, 27). It appears that one of the roles of activin in the ovary is to stimulate smaller follicles

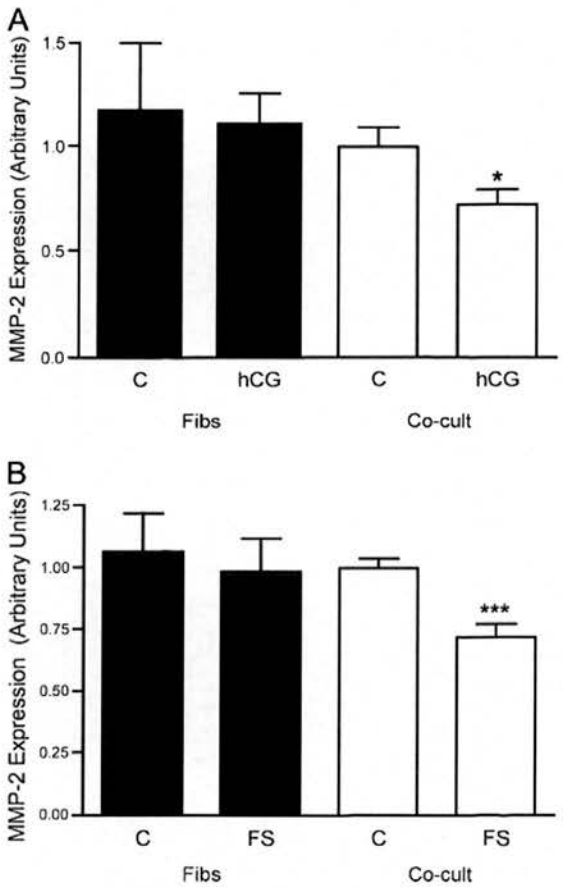

FIG. 5. *In vitro* model of paracrine signaling in coculture experiments of luteinized granulosa cells and luteal fibroblast-like cells. A, MMP-2 expression was significantly decreased ($P < 0.05$, by t test) in primary cocultures exposed to hCG (100 ng/ml) for 24 h; B, similarly, the expression of MMP-2 was significantly reduced ($P < 0.01$, by t test) in cocultures treated with follistatin (FS) (500 ng/ml) for 24 h. Fibroblast-like cell cultures in both treatment groups were unaffected by either hCG or follistatin ($P > 0.05$, t test).

and inhibit luteinization of larger follicles to maintain the follicle in an FSH-responsive state (24, 28).

There is evidence to support a paracrine role for activin in the corpus luteum. In the first instance, the corpus luteum has the mechanism to synthesize and secrete activin. Studies in women (29) and primates (30) have localized inhibin α and βA subunit mRNA and protein to the steroidogenic cells of the corpus luteum. Dispersed luteal cells *in vitro* and the intact corpus luteum *in vivo* secrete inhibin-A in a regulated manner (31). The same is seen in cultures of human luteinized granulosa cells. In addition, these cells secrete activin-A, and activin-A is found in the follicular fluid at the time of ovulation (18). Indeed, circulating activin concentrations change across the ovarian cycle, with an increase toward the end of the luteal phase (10). As well as expressing the βA subunit mRNA and protein, it is likely that the granulosa-lutein cells of the corpus luteum secrete activin-A during the luteal phase.

In addition, we have shown that the corpus luteum has the molecular mechanisms to respond to locally produced activins. Activins signal through two types of transmembrane serine/threonine kinase receptor interactions (32) and the cytoplasmic to nuclear translocation of the intracellular Smad proteins (33). Human corpora lutea express both the type I (ALK 2/4) and type II (A) activin receptors as well as components of the Smad (2, 3, and 4) signaling pathway that are induced by activin. Correspondingly, we have localized the receptors and activated nuclear phosphorylated Smad 2/3 to both steroidogenic and stromal cells of the human corpus luteum. Although the nature of the ligand signaling through the Smad 2/3 pathway is not entirely clear, because TGF-β signals through similar Smads (32, 34), it is likely that the activin signaling cascade is active and that activins do act locally on different cells types in the corpus luteum.

Activin action, however, is highly controlled in physiological systems. It is tightly regulated by various inhibitors at the ligand, receptor, and postreceptor levels (35). Follistatin, a local regulator of activin, controls activin signaling by forming biologically inactive complexes with the β-subunits of the activin glycoprotein. Suppression of activin-regulated processes is also achieved by the activin antagonist inhibin and its coreceptor β-glycan. Inhibin opposes activin action by competitively binding to the same site of the type II activin receptor. Additionally, inhibins have also been thought to have interactions with specific and high-affinity receptors that may then activate signal transduction pathways, such as the inhibitory Smads, that can oppose activin action (36). β-Glycan on the other hand, has a high affinity for inhibin and forms complexes with the type II activin receptor to block the recruitment of the type I receptor (37) that is required to activate the signaling cascade. As well as making activin, the corpus luteum has at least some of the molecular mechanisms required to inhibit activin.

It is not clear whether activin signaling changes across the luteal phase. It is likely there is more activin available to act in the late luteal phase because the increase in circulating activin-A at the time of luteolysis (10) is not seen with regard to its inhibitors, inhibin A (10, 31) and follistatin (38). We were able to immunolocalize nuclear phosphorylated Smad 2/3, which would be detected in the presence of activin signaling, throughout the luteal phase. Although most increases in Smad signaling are through phosphorylation of the proteins, there are also increases in the expression of Smad mRNA in response to ligand activation. We therefore investigated the expression of Smad 2 and 3 mRNA across the luteal phase. Expression of both Smads tended to be maximal in the late-luteal phase. These results are compatible with activin action increasing in the lead up to luteolysis.

The expression of both activin and its local inhibitors is regulated in the corpus luteum. The addition of hCG upregulates the inhibin α-subunit and the secretion of inhibin-A from steroidogenic cells *in vitro* and *in vivo* (31, 39). We hypothesized that the inhibin:activin ratio would increase in the presence of hCG and tested this using luteinized granulosa cells in culture. We showed a similar stimulation of both activin-A and inhibin-A and no change to the ratio. However, other studies have shown that short-term gonadotropin stimulation initially increased activin-A secretion, but unlike inhibin-A, this diminishes later in a time- and dose-dependent manner (40), and it is possible that hCG increases the inhibin:activin ratio in the longer term. In ad-

dition, both bound inactive activin and free active activin were detected by the assay, and it is possible that the ratio of inhibin to active activin changes. This is particularly important because it is clear that follistatin is hormonally regulated. hCG up-regulates follistatin from luteinized granulosa cells in culture, and follistatin concentrations in follicular fluid are hormonally regulated (38). For instance, follistatin is up-regulated in the serum of pregnant women (15). Collectively, these data are suggestive that luteal activin action, although not necessarily secretion, is inhibited by hCG during luteal rescue.

There appears to be a role for increased activin activity during luteolysis. Previously, activin has been shown to inhibit progesterone production by luteal cells *in vitro* (41), and therefore it may have a role in the changes associated with functional luteolysis. However, here we suggest that activin has a role in the remodeling associated with structural luteolysis. One of the key features associated with luteolysis is the up-regulation of MMP-2 in stromal cells (5, 6, 42). We have shown for the first time that fibroblast-like cell MMP-2 is up-regulated by concentrations of activin (but not inhibin) found inside the human ovary. For clarity, we use the term fibroblast-like cells and stroma to represent the stromal cells that are directly surrounding the steroidogenic cells and invaginating in between them. Furthermore, stromal MMP-2 expression can be inhibited by hCG in a paracrine manner both *in vivo* (5) and in coculture models *in vitro*. The effect of hCG on MMP-2 in these cocultures can be replicated by the addition of follistatin at concentrations found within the human ovary. Because hCG up-regulates follistatin, these results suggest that activin-A may be involved locally in the physiological regulation of MMP-2 activity in the corpus luteum.

MMP-2 is not known for being regulated at the level of its expression. Indeed, an examination of the promoter region of the MMP-2 gene shows fewer regulatory sequences than other MMPs (42), consistent with a gene that is normally constantly expressed. However, there are clearly certain circumstances when its expression is regulated. It is increased in fetal membranes during labor (43) and in the endometrium during menstruation (44), and more importantly, it is increased during natural and induced luteolysis in a wide range of different species (5, 6, 42). The factors involved in this regulation are not certain. There has been a suggestion that steroids are involved in its regulation in the endometrium (45) and cytokines such as TNF-α may be important in the regulation of endometrial fibroblast (46) and bovine luteal (47) MMP-2 expression. Extravillous trophoblasts overexpressing Smad 4 demonstrate a clear up-regulation of MMP-2 production, mimicking the effect of TGF-β in this particular system (48). The same theory may apply to activin-A in fibroblast cells of the corpus luteum because we know they express the Smad proteins common to both TGF-β and activin signaling and that activin-A in fibroblast-like cells increases MMP-2 production. Here, we demonstrate that activin-A can up-regulate luteal MMP-2 expression and activity *in vitro* at physiological concentrations.

There is some evidence, in other systems, that activin may well regulate MMP-2 expression. Activin-A has been found to regulate MMP-2 in both mouse peritoneal macrophages (49) and human decidua (50, 51). In addition, it has been suggested that activin can up-regulate both MMP-9 and MMP-2 expression in cultured human cytotrophoblast cells (50). However, this was thought to be secondary to activin affecting the differentiation status of these cells. Another molecule from luteal fibroblasts with a role in tissue remodeling during luteolysis is connective tissue growth factor (3). We have shown that activin also up-regulates its expression in luteal fibroblast-like cells (3), although this does not appear to be the only regulating factor. It is not clear whether other molecules as well as activin can regulate luteal MMP-2 expression during luteolysis.

If activin-A is involved in regulating tissue remodeling

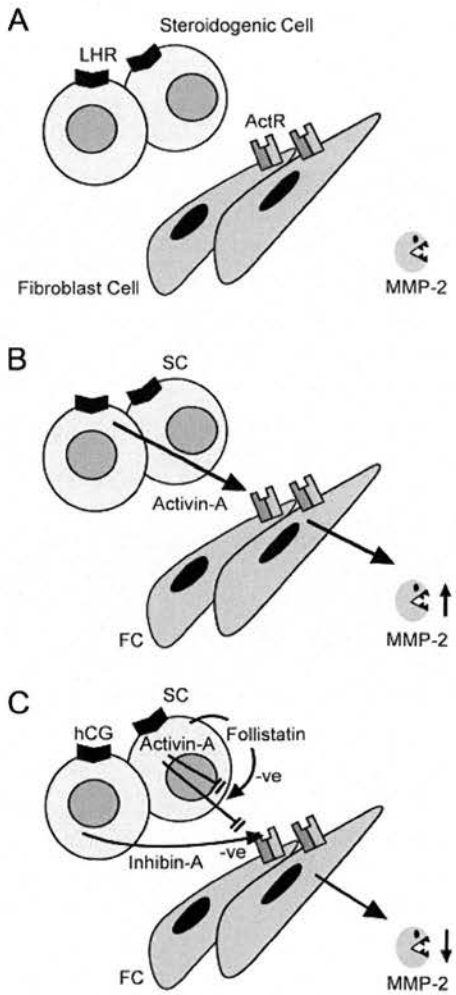

FIG. 6. Schematic of the model we propose for the paracrine regulation of MMP-2 expression by hCG in human luteal cells. A, Steroidogenic cells (SC), but not fibroblast-like cells (FC), express the LH/hCG receptor. The fibroblast cells secrete MMP-2 and express activin receptors. B, During the late-luteal phase, increasing activin-A action from the steroidogenic cells up-regulates the expression of MMP-2 from the fibroblast cells. C, In the presence of hCG during maternal recognition of pregnancy, the increase of inhibin-A will block activin binding to its receptors, whereas follistatin will bind and biologically inactivate secreted activin-A, resulting in decreased activin signaling, thus preventing the increased expression of MMP-2. We suggest that activin-A is a paracrine signaling molecule playing a major role in the regulation of luteolysis in women.

during luteolysis, it is likely to be self-limiting in nature. This is because the cellular source of activin is the steroidogenic cells that undergo programmed cell death when the corpus luteum is cleared from the ovary (1, 2). It is difficult to determine whether observations made in nonprimate species are relevant to women because there are major species differences in the regulation of the corpus luteum. In the mouse, where ovarian activin βA expression has been conditionally knocked out (52), the ovary contains multiple corpora lutea when examined. Although is it tempting to speculate that luteolysis may be affected, it has to be remembered that inhibin-A will also be knocked out, and this may well result in higher FSH concentrations than would be expected to cause more ovulations.

Observational studies on human corpora lutea inform us that the steroidogenic cells expressing LH/hCG receptors can secrete activin-A, and the stromal cells that express MMP-2 have the molecular machinery to respond to locally produced activin. Such observations, however, do not tell us the roles of activin, and interventional studies are required. It is now not practical to collect enough well characterized human corpora lutea to disaggregate the cells and manipulate these cells in culture, and other model systems have to be used. The culture of luteinized granulosa cells and the derivation of fibroblast-like cells have the potential to replicate the luteal steroidogenic cell/stromal cell interactions *in vitro*. Although luteinized granulosa cells are LH/hCG responsive and function in primary culture for 14 d, they may differ from mature granulosa-lutein cells. In addition, although the phenotype of the fibroblast-like cells is identical to luteal stromal cells in all the genes we have analyzed (3) and expression patterns of MMP-2 from prolonged cultures of fibroblasts obtained from corpora lutea, there may be differences. Indeed, we have shown that there are at least two types of fibroblast in the human corpus luteum (53). Furthermore, in culture, the cells do not have the local interaction with immune cells and endothelial cells seen *in vivo*.

Although we accept there are caveats to our model system and indeed, our *in vitro* cell culture and coculture models may not entirely mimic what is happening within the corpus luteum, such model systems can give valuable insights into mechanistic functions that otherwise may not be dissected. Therefore, although care needs to be used in the interpretation of their findings and used in conjunction with observational studies, we believe we have the most appropriate model to study and manipulate cell-cell interactions in the human corpus luteum.

In conclusion, we suggest that activin-A may have a physiological role in luteolysis, and part of this role is to stimulate luteal MMP-2 expression. We believe that hCG serves to impede activin action (Fig. 6), and this will facilitate luteal maintenance by inhibiting luteolysis and allowing the maternal recognition of pregnancy.

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Role of Luteal Glucocorticoid Metabolism during Maternal Recognition of Pregnancy in Women

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The human corpus luteum (hCL) is an active, transient, and dynamic endocrine gland. It will experience extensive tissue and vascular remodeling followed by 1) demise of the whole gland without any apparent scarring or 2) maintenance of structural and functional integrity dependent on conceptus-derived human chorionic gonadotropin (hCG). Because cortisol has well-characterized roles in tissue remodeling and repair, we hypothesized that it may have a role in controlling luteal dissolution during luteolysis and would be locally produced toward the end of the luteal cycle. Glucocorticoid-metabolizing enzymes [11β-hydroxysteroid dehydrogenase (11βHSD) types 1 and 2] and the glucocorticoid receptor (GR) were assessed in hCL and cultures of luteinized granulosa cells (LGC) using immunofluorescence and quantitative RT-PCR. Furthermore, the effect of cortisol on steroidogenic cell survival and fibroblast-like cell activity was explored *in vitro*. The hCL expressed 11βHSD isoenzymes in LGC and nuclear GR in several cell types. hCG up-regulated the expression and activity of 11βHSD type 1 ($P < 0.05$) and down-regulated type 2 enzyme ($P < 0.05$) *in vitro* and tended to do the same *in vivo*. Cortisol increased the survival of LGC treated with RU486 ($P < 0.05$) and suppressed the activity of a proteolytic enzyme associated with luteolysis in fibroblast-like cells ($P < 0.05$). Our results suggest that, rather than during luteolysis, it is luteal rescue with hCG that is associated with increased local cortisol generation by 11βHSD type 1. Locally generated cortisol may therefore act on the hCL through GR to have a luteotropic role in the regulation of luteal tissue remodeling during maternal recognition of pregnancy. (*Endocrinology* 148: 5769–5779, 2007)

THE HUMAN CORPUS LUTEUM develops from the postovulatory follicle to become one of the most active endocrine glands in the body. During the luteal life span, this remarkable structure will experience very high cell turnover, intense angiogenesis, and considerable steroid production within a 14-d period. Such highly organized events rely upon stringent paracrine interactions between neighboring and surrounding cells. In a nonconception cycle, the corpus luteum will undergo luteolysis, a complex process whereby the tissue is subjected to a loss of structural and functional integrity that is associated with an immune cell influx (1), increase in matrix metalloproteinases (2), cell death (3), and vascular regression (4). Remarkably, however, such dramatic remodeling and rapid tissue removal occurs in an orderly fashion without any evidence of permanent scar tissue.

Within the ovary, ovulation and the resulting folliculoluteal transition has been likened to an inflammatory response, as a result of the acute hemodynamic, cellular, and biochemical changes that occur at the site of follicle rupture (5, 6). Key studies have clearly shown that the repetitive damage from consecutive ovulations must be quickly repaired in anticipation for the next ovulatory cycle, and locally produced glucocorticoids are involved (7–9). Cortisol, the most important glucocorticoid, is well known to minimize inflammatory damage to tissues by encouraging wound healing and subsequent repair. Clear evidence of this phenomenon is apparent from the switch of 11β-hydroxysteroid dehydrogenase (11βHSD) isoforms over the ovarian cycle, complemented by the increased concentrations of cortisol in follicular fluid after the LH surge (10). At present, both 11βHSD type 1 (that tends to generate cortisol) and 11βHSD type 2 (that tends to inactivate cortisol) isoforms, and their activities, have been reported in the ovaries of several species (11).

Throughout the human body, glucocorticoids are well known to exhibit a plethora of physiological roles. Although most of these actions have been best characterized in body systems such as kidney, liver, adrenal, and local inflammatory responses, influences on human fertility, oocyte maturation, and the establishment of a functional corpus luteum have been suggested (12–15). It has also been reported that because glucocorticoids can inhibit endothelial cell proliferation (16), they may have a role in the local regulation of angiogenesis.

With the knowledge that glucocorticoids are involved in inflammatory-associated events in the ovary and may adversely affect angiogenesis, we hypothesized that local cortisol action may have a role in the luteolytic process. Although governed by different luteolytic mechanisms than in women, elegant studies in the rat corpus luteum have identified a potential role for 11βHSD type 2 in the regressing corpus luteum (17). Therefore, based upon the findings in the

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Abbreviations: CBP, Cortisol-binding protein; GR, glucocorticoid receptor; hCG, human chorionic gonadotropin; 11βHSD, 11β-hydroxysteroid dehydrogenase; LDL, low-density lipoprotein; MMP-2, matrix metalloproteinase 2; NGS, normal goat serum; TBS, Tris-buffered saline.

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rat study, coupled with much evidence for glucocorticoids in tissue and scar regeneration, we hypothesized that glucocorticoids were involved across the luteal life span. Therefore, the aims of the current study were to 1) investigate luteal glucocorticoid metabolism, reception, and subsequent regulation using carefully dated human corpora lutea and primary cell cultures of luteinized granulosa cells and 2) establish whether changes in cortisol synthesis and metabolism might be a key event in the regulation of the tissue remodeling associated with luteolysis.

Materials and Methods

Collection of human corpora lutea

Tissue collection was approved by the local medical research ethics committee, and all women gave informed consent. Human corpora lutea (n = 18) were enucleated at the time of surgery from women with regular menstrual cycles undergoing hysterectomy for benign conditions and dated on the basis of the urinary LH surge as described previously (18, 19). In this study, six corpora lutea were classified as early luteal (LH+1 to LH+5), six as mid-luteal (LH+6 to LH+10), and six as late-luteal (LH+11 to LH+14). At operation, the corpus luteum was quartered to ensure that each quarter contained all cellular elements and fixed in 10% neutral buffered formalin for subsequent immunohistochemistry. In addition, archival corpora lutea that had been immediately frozen and stored at −70 C from previous studies (20, 21) were also available. Frozen tissue quarters for mRNA extraction were available from six late-luteal corpora lutea and five corpora lutea that had been rescued (daily doubling doses of exogenous human chorionic gonadotropin (hCG) from LH+7 for 5–8 d) as described previously (2, 19).

Isolation of human luteinized granulosa cells and fibroblast-like cells

The medical ethics committee separately approved the collection of cells from patients undergoing assisted conception. With patient consent, follicular fluid was collected from women undergoing transvaginal oocyte retrieval for *in vitro* fertilization after ovarian stimulation using a standard procedure (22). Isolation of luteinized granulosa cells using Percoll density gradient centrifugation was carried out as described previously (20, 23). Fibroblast-like cells were obtained from prolonged cultures of follicular aspirates as described previously (20).

Primary cell culture treatments

Pooled luteinized granulosa cells (100,000 per well of three to five patients) were cultured in 24-well plates precoated with Matrigel (BD Biosciences, Bedford, MA) in serum-free medium (supplemented DMEM/Ham's F12 mixture), as described previously (20). Each pooled experiment for the following treatments was carried out at least three times to avoid biological bias.

Assessment of the acute effects of hCG and progesterone

Luteinized granulosa cells plated as above had fresh medium changed every 2–3 d over the course of culture, and treatment was carried out on d 6 or 7 of culture. The treatments groups were 1) controls with low-density lipoprotein (LDL, 50 mg/liter; Sigma-Aldrich Corp., Dorset, UK), 2) hCG (10 ng/ml; Serono, Welwyn Garden City, UK) with LDL (50 mg/liter), and 3) hCG (10 ng/ml) and LDL (50 mg/liter) in conjunction with 100 μM aminoglutethimide (Sigma-Aldrich). After 24 h, medium and cells were stored for steroid analysis and mRNA extractions, respectively. Progesterone concentrations in the culture media were determined by an in-house RIA as described previously (20).

Manipulation of hCG in prolonged cultures of luteinized granulosa cells

To mimic the luteal phase in primary cell culture, luteinized granulosa cells were plated as described above and grown for 12 d as described previously (22). Briefly, cells were stimulated with low-dose hCG (1 ng/ml) with LDL (50 mg/liter) beginning on d 2 and this was repeated every second day until d 7 when treatments were replaced with maximal doses of 100 ng/ml hCG/LDL or LDL alone. Cells were analyzed after 7 d with hCG and on d 12 in the presence or absence of hCG to mimic the progesterone secretion profile of late-luteal and luteal rescue stages, respectively (22).

Relative cell counts for various steroid and steroid inhibitor treatments

Pooled luteinized granulosa cells were cultured as above for 7 d in the presence of the same carrier ethanol concentrations in each well. The experiments were piloted to determine the appropriate concentrations of reagents, and final experiments were repeated three times in triplicate. The treatments were 1) control, 2) aminoglutethimide (100 μM), 3) RU486 (100 μM; Sigma), 4) RU486 (100 μM) with progesterone (500 μM; Sigma), 5) RU486 (100 μM) with cortisol (500 μM; Sigma), and 6) RU486 (100 μM) with hCG (100 ng/ml). After 7 d, the cells were removed by trypsinization, resuspended, and counted using a hemocytometer. Values were taken as the mean of at least four separate counts by an observer blinded to the treatments and related to controls.

Treatment of cultured fibroblast-like cells with cortisol

Cultures of fibroblast-like cells were derived as described above and transferred to 24-well plates at a concentration of 60,000–80,000 cells per well. After 6 h in serum-free culture, the medium was removed and replaced with medium containing either cortisol (100 nM) or an equivalent amount of the ethanol carrier as a control. After 24 h, the culture medium was collected for subsequent zymography, and the cells were used for mRNA extraction.

Preparation of cDNA from human corpora lutea and cultured cells

Messenger RNA was batch extracted from frozen human corpora lutea and reverse transcribed into cDNA using random hexamers as described previously (20). Luteinized granulosa cell mRNA was extracted using RNeasy mini-spin columns after lysis by the addition of RNeasy lysis buffer (QIAGEN, Crawley, Sussex, UK). Lysates were frozen until processed as per manufacturers' protocols and then DNase treated with on-column DNase I (QIAGEN) and quantified using the NanoDrop ND-1000 Spectrophometer (NanoDrop Technologies, Wilmington, DE). mRNA was then reverse transcribed into cDNA using random hexamers (Applied Biosystems, Foster City, CA).

Quantitative analysis of gene expression by real-time PCR

Quantitative real-time PCR was carried out on the ABI PRISM 7700 sequence detection system (Applied Biosystems) using specific primers and probes (Eurogentec, Southampton, UK) for each gene of interest

TABLE 1. List of all primer/probe sequences used for Taqman quantitative RT-PCR

Gene	Forward primer 5′–3′	Reverse primer 5′–3′	Probe 5′-FAM-TAMRA-3′
GRα	GCGATGGTCTCAGAAACCAAAC	GCAGAGGATAACTTCCTCTGTAATCTC	TCAGAGCCTCAGCAACCTTCACTGCA
11βHSD1	AGCTCTGCGCCAAGAAGAAGT	AGGATCTTCCTGCATGGATTTC	TGACAGCTCACTCTGGACCACTCTTCTGA
11βHSD2	GGCCAAGGTTTCCCAGTGA	GTTGTGCCAGGAGGGTGTTT	CTCTGCGCCTCTCCACTGTTTCATGA
MMP-2	TTCCTGGGCAACAAATATGAGA	TGGTCGCACACCACATCTTT	AGCGCCGGCCGCAGTGA

Sequences for 11βHSD1, 11βHSD2, and GRα were obtained from Rae *et al.* (8).

(Table 1), and levels were related to a ribosomal 18S internal control (Applied Biosystems). Quantitative real-time PCR was performed with an extension temperature of 72 C and 30 cycles of amplification. All samples were performed in duplicate, and a relative comparison was made to an appropriate tissue control, either human placental or liver cDNA.

Histology

Masson's modified trichrome staining was carried out on 5-μm paraffin sections of human corpora lutea. Briefly, sections were dewaxed, rehydrated in alcohol, and washed in water. Sections were then placed in Celestine blue for 3 min, washed, placed in Mayer's acid hematoxylin solution for 3 min, washed, and then counterstained with Biebrich scarlet-acid fuchsin solution for 15 min. After washing, the sections were differentiated in 5% phosphotungstic acid for 15 min before being placed directly into aniline blue solution for 5–10 min, washed, dehydrated, and mounted for microscopy.

Immunohistochemistry

Immunolocalization of glucocorticoid receptor (GR) was carried out using a rabbit polyclonal antibody (ABR; Cambridge BioScience, Cambridge, UK) in 5-μm paraffin tissue sections of human corpora lutea prepared on poly-L-lysine-coated microscope slides. These sections were dewaxed, rehydrated, washed in PBS, subjected to microwave antigen retrieval in 0.01 M citric acid (pH 6.0) for 10 min, and left to cool to room temperature. All sections were washed and placed in 3% H_2O_2/methanol for 30 min, followed by an avidin and biotin block and another block using normal goat serum (NGS; Diagnostics Scotland, Edinburgh, UK) diluted 1:4 in PBS containing 5% BSA [NGS/Tris-buffered saline (TBS)/BSA] for 1 h at room temperature.

GR antibody was diluted 1:2000 in blocking solution and incubated on sections overnight at 4 C. After rinsing, sections were incubated with the biotinylated goat antirabbit IgG (diluted 1:500 in NGS/TBS/BSA) secondary antibody (Dako Corp., Cambridge, UK) for 1 h. After washing, the sections were incubated in avidin-biotin complex-horseradish peroxidase (Dako), and binding was visualized by incubation with liquid 3,3′-diaminobenzidine tetrahydrochloride (Dako). Sections were counterstained lightly with hematoxylin to enable cell identification. Negative controls were performed identically to the above protocol with primary antibody incubations substituted with blocking serum containing nonspecific Igs at the same concentration. Images were captured using an Olympus Provis microscope (Olympus Corp. Optical Co., London, UK) equipped with a Kodak DCS330 camera (Eastman Kodak Co., Rochester, NY), stored on an HP computer and assembled using Photoshop 7.0.1 (Adobe, Mountain View, CA).

Fluorescent immunohistochemistry

Sections were washed, subjected to antigen retrieval, and blocked as described above, and negative controls were performed as above. Rabbit anti-GR and rabbit anti-11βHSD type 1 (Cayman/IDS Ltd., Bolton UK) diluted 1:100 in NGS/TBS/BSA were incubated on sections overnight at 4 C. Sections were washed, and slides were incubated with goat antirabbit IgG 488 (Dako) diluted 1:200 in PBS for 1 h.

Sections that were labeled with anti-GR were subjected to further colocalization experiments. Sections were reblocked with NGS/PBS/BSA for 1 h and then incubated with mouse monoclonal antibodies anti-CD31 (Dako; 1:20 in block), anti-CD68 (Dako; 1:20 in block), or anti-α-smooth muscle actin (α-SMA, Dako; 1:500 in block) overnight at 4 C. Sections were washed and incubated with the fluorochrome streptavidin 546 Alexafluor (Molecular Probes, Eugene, OR) diluted 1:200 in PBS for 1 h.

Sections that were labeled with anti-11βHSD type 1 were reblocked with normal donkey serum/PBS/BSA for 1 h and then incubated with sheep anti-11βHSD type 2 (kind gift from Prof. Ian Mason, The University of Edinburgh, Edinburgh, UK) diluted 1:50 in donkey serum. Sections were washed and incubated with donkey antisheep peroxidase (Dako) 1:200 in normal donkey serum/PBS/BSA for 30 min before washing and incubating for 10 min with tyramide Cy3 (TSA plus cyanine 3 system; PerkinElmer Life Sciences, Boston, MA) diluted 1:50 in the supplied buffer to amplify the 11βHSD type 2 immunostaining with red fluorescence.

Nucleic acids were labeled with To-Pro 3 and washed and mounted in Permafluor (Beckman Coulter, High Wycombe, UK). Fluorescent images were captured using an LSM 510 Axiovert 100M confocal microscope (Carl Zeiss Ltd., Welwyn Garden City, UK). Images of 11βHSD type 1 and 11βHSD type 2 were analyzed comparatively by standardizing the computer settings for each isoform. Therefore, the relative intensity of staining for each isoform corresponds to abundance of protein levels. All images were compiled using Photoshop 7.0.1 (Adobe Systems Inc., San Jose, CA).

Measurement of net 11βHSD oxidoreductase activity

Interconversion of cortisone to cortisol via 11-oxoreductase activity was assessed in the presence and absence of hCG. Pooled luteinized granulosa cells were stimulated with either 100 ng/ml hCG in serum-free medium or serum-free medium alone for 24 h. Controls included incubations containing no cells with only Matrigel. After hCG stimulation, culture medium was discarded from wells and replaced with culture medium containing 100 nM cortisone substrate and 0.1 μCi [^{3}H]cortisone to give a final volume of 500 μl/well. All incubations were in triplicate for 4 h at 37 C with 95% air-5% CO_2. After incubation, medium was added to glass tubes containing 5-ml aliquots of dichloromethane and vortexed thoroughly. To separate the aqueous and organic phases, tubes were centrifuged at 12,000 rpm for 10 min. After the aqueous phase was removed, samples were evaporated to dryness under nitrogen gas at 45 C. Steroid residues were resuspended in 100 μl dichloromethane and samples along with one [^{3}H]cortisol and one [^{3}H]cortisone blot were transferred to silica gel-precoated plastic sheets for thin-layer chromatographic separation of precursor and product in the solvent system of chloroform-ethanol (92:8 by volume) (Merck, Haddeson, Hertfordshire, UK). Thin-layer chromatography plates were then scanned using a Bioscan System 200 detector (Lablogic Systems, Sheffield, UK), and corresponding peaks were analyzed for enzymatic activity in each sample and consequently each treatment group. Results are expressed as amount of cortisone converted to cortisol (picomoles) per hour.

Gelatin zymography

Cell culture medium was collected from serum-free cultures and subsequently frozen at −20 C. Aliquots of 200 μl were subjected to freeze drying for 2–3 h until they resembled a powder and then reconstituted in 20 μl sterile dH_2O. One microliter of the reconstituted sample was added to sample buffer [10% (vol/vol) glycerol, 1% (wt/vol) SDS, and 0.04% (vol/vol) bromophenol blue] and applied to an 11% (wt/vol) polyacrylamide gel containing 1 mg/ml gelatin and 0.1% (wt/vol) SDS. Gels were incubated in 2.5% Triton X-100 for 45 min after electrophoretic separation of proteins and then incubated at 37 C overnight in digestion buffer [50 mmol/liter Tris-HCl (pH 7.6) containing 0.2 mol/liter NaCl, 5 mmol/liter $CaCl_2$, and 0.02% (wt/vol) Brij 35] as described previously (2). Gels were stained in staining solution [30% (vol/vol) methanol, 10% glacial acetic acid, and 0.5% (wt/vol) Coomassie brilliant blue G250] and then destained in the same solution minus the Coomassie staining dye. The bands on the zymography gels reflect the activity of matrix metalloproteinase 2 (MMP-2) and were analyzed by transmission densitometry (G-700 densitometer; Bio-Rad, Hemel Hempstead, Hertfordshire, UK), and integrated software (Quantity One, Bio-Rad). All densitometry measurements were made between samples on the same gel or between gels run under identical conditions with a common control sample on each gel to ensure comparability.

Statistical analysis

Parametric statistics were used when the data were distributed normally, with appropriate tests highlighted in the figure legends. Differences were considered significant at $P < 0.05$ level.

Results

Expression and localization of 11βHSDs in the human corpus luteum

The granulosa-lutein cells of the corpus luteum can easily be identified by their localization and morphological characteristics (Fig. 1A). Both isoforms of the cortisol-metabolizing enzyme 11βHSD (type 1 and type 2) could be immunolocalized to the cytoplasm of granulosa-lutein cells in human corpora lutea (Fig. 1, B and C), although the staining intensity of 11βHSD type 1 was much greater than that of 11βHSD type 2. Specific immunostaining for each isoform could be detected in each corpus luteum analyzed from each stage of the functional luteal phase. No staining could be detected in negative control sections (Fig. 1B, *inset*). The corpus luteum of women therefore has the capacity for the local production and metabolism of cortisol.

Expression and localization of genomic GR in the human corpus luteum

Nuclear GR immunostaining could also be detected in human corpora lutea. GR were also localized to the nuclei of granulosa-lutein cells (Fig. 1D), but unlike the 11βHSD isoforms, GR could be localized to nonsteroidogenic cell types. Dual-staining immunohistochemistry with α-SMA demonstrated GR expression in stromal myofibroblasts and pericytes (Fig. 1, E and F). Dual immunostaining with CD68 and CD31 demonstrated specific nuclear GR expression in macrophages (Fig. 1G) and endothelial cells (Fig. 1H) in each corpus luteum examined. This pattern of GR immunostaining was detected in corpora lutea from each stage of the luteal phase. Various different cells in the human corpus luteum therefore have the capacity to respond to both systemic and locally produced cortisol in an autocrine, paracrine, or endocrine manner.

The effect of hCG on 11βHSD and GR expression in primary cultures of luteinized granulosa cells

To determine whether the expression of GR and cortisol-metabolizing enzymes in steroidogenic cells could be acutely regulated, the effect of hCG was examined in primary cell cultures of human luteinized granulosa cells. The addition of hCG for 24 h resulted in a 30-fold up-regulation of 11βHSD

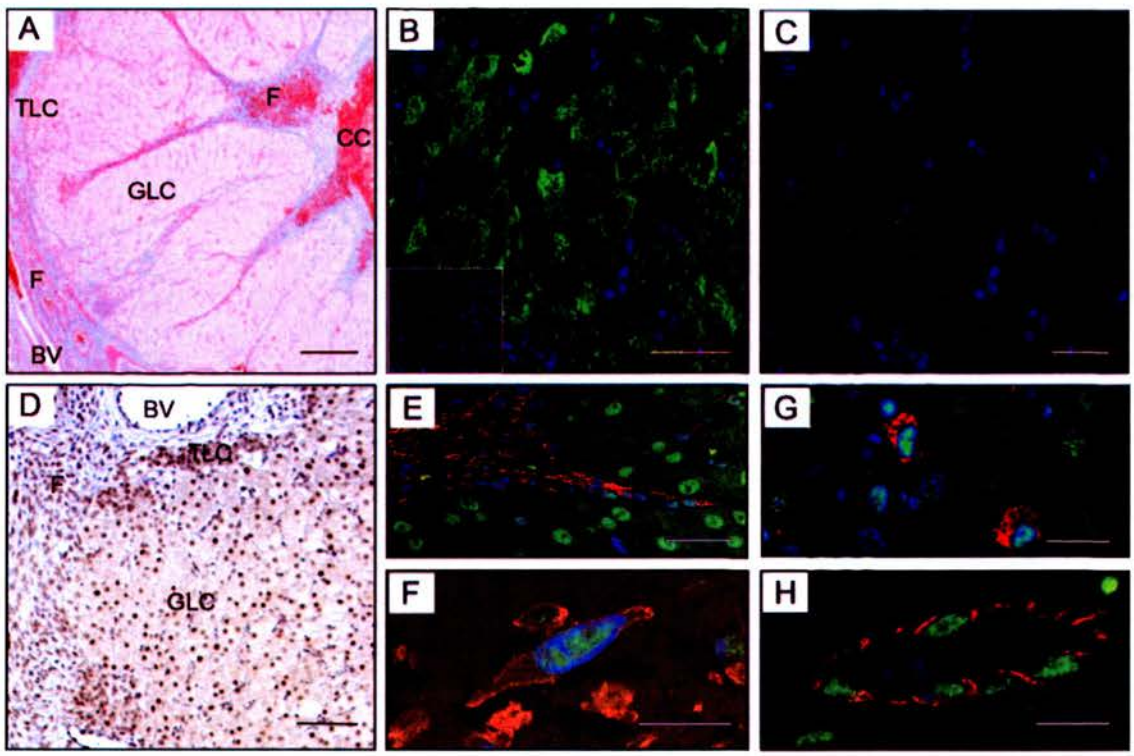

FIG. 1. Immunolocalization of cortisol-metabolizing enzymes and the GR in the human corpus luteum. A, A midluteal human corpus luteum (stained with Masson's trichrome) is a heterogeneous tissue composed of the steroidogenic granulosa-lutein cells (GLC) and theca-lutein cells (TLC), an inner and outer supportive stromal layer that consists of predominantly fibroblast (F) layers and blood vessels (BV), and the central clot (CC) that once comprised the antral cavity of the follicle; B and C, immunofluorescence staining demonstrates that the GLC of the corpus luteum express the cortisol-metabolizing enzymes 11βHSD type 1 (*green* with *blue* nuclear stain) (B) and 11βHSD type 2 (*red* with *blue* nuclear stain) (C); *inset* in B, negative control for 11βHSD type 1 and 2; D, the GR protein is ubiquitously expressed in the corpus luteum as seen by the positive *brown* DAB staining; E, immunofluorescence, however, further confirms that GR can be colocalized with other cell types; for example, nuclear GR (*green*) is present in the GLC, whereas α-SMA-positive cells represent stromal myofibroblasts and pericytes (*red*); F, high-power images reveal that GR (*green*) is localized to the presumptive fibroblast cells (*red*); G and H, macrophages (*red*, CD68) (G) and endothelial cells (*red*, CD31) (H) of the corpus luteum. Nuclear staining is depicted in *blue*. *Scale bars*, 200 μm (A), 40 μm (B, C, and E), 100 μm (D), 10 μm (F and H), and 20 μm (G).

type 1 expression ($P < 0.05$, Kruskal-Wallis) (Fig. 2A). At the same time, 11βHSD type 2 was down-regulated ($P < 0.05$, Kruskal-Wallis) (Fig. 2B), whereas GR expression was also up-regulated ($P < 0.05$, ANOVA) (Fig. 2C). To determine the functionality of the enzyme and the direction of 11βHSD activity, 11-oxoreductase activity was assessed in the presence and absence of hCG. This confirmed that hCG stimulated reductase activity and acted to generate cortisol ($P < 0.05$, *t* test) (Fig. 2E).

Because luteinized granulosa cells have genomic progesterone receptors, and hCG stimulates progesterone secretion (range, 460–890 ng/ml), cells were cultured with the progesterone synthesis inhibitor aminoglutethimide to separate hCG effects from progesterone effects (20) ($P < 0.0001$, ANOVA) (Fig. 2D). Aminoglutethimide reduced progesterone (range 160–400 ng/ml) secretion to control levels (170–200 ng/ml) in the presence of hCG (Fig. 2D). Although aminoglutethimide had no effect on hCG-stimulated up-regulation of 11βHSD type 1 and GR, it inhibited the hCG-induced down-regulation of 11βHSD type 2 (Fig. 2B). This suggests that progesterone may be involved in the local regulation of 11βHSD isoform expression. Overall, these data suggest that cortisol metabolism and reception may be hormonally regulated in luteal steroidogenic cells.

The effect of chronic manipulation of hCG in cultures of human luteinized granulosa cells

To determine whether hCG could regulate the expression of 11βHSDs and GR in more physiologically relevant prolonged culture conditions, 12-d cultures of luteinized granulosa cells, designed to mimic the luteal phase (20), were examined. Withdrawal of hCG in culture down-regulated 11βHSD type 1 ($P < 0.05$, Kruskal-Wallis), whereas its expression was maintained in the presence of hCG (Fig. 3A). Conversely, hCG withdrawal had no effect on 11βHSD type 2 expression, whereas maintenance of hCG did not alter its expression because the trend toward reduction did not reach significance ($P > 0.05$, Kruskal-Wallis) (Fig. 3B). The expression of GR showed a similar pattern to that of 11βHSD type 1, but there were no significant changes detected (Fig. 3C) ($P > 0.05$, ANOVA). These data suggest that cortisol-metabolizing enzymes may continue to be differentially regulated by hCG under chronic conditions.

The effect of hCG on the expression of 11βHSDs in human corpora lutea in vivo

To determine the effect of hCG of luteal 11βHSD expression in women, we examined archival tissues collected in the late luteal phase in the absence or presence of exogenous hCG to rescue the corpus luteum and mimic the changes of

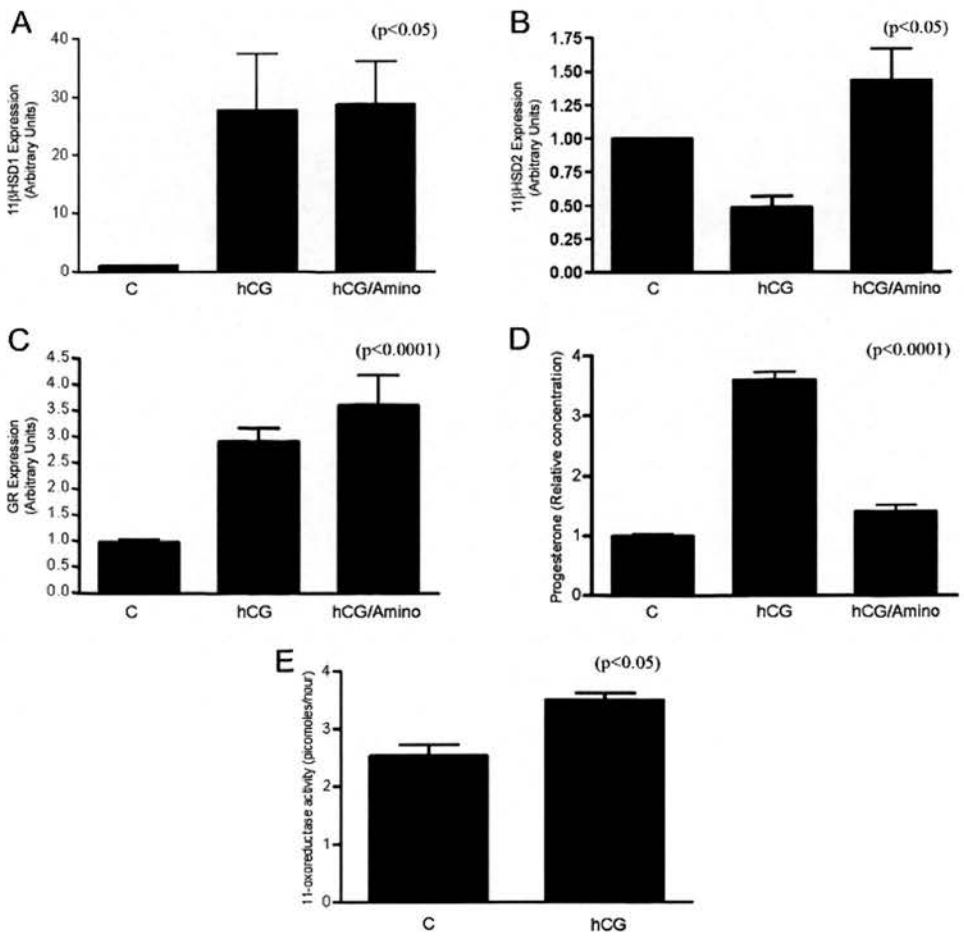

FIG. 2. hCG modulates the 11βHSD enzymes and GR in luteinized granulosa cells in primary culture. A, The expression of 11βHSD type 1 is up-regulated by hCG independently of progesterone as evident by the addition of progesterone synthesis inhibitor aminoglutethimide ($P < 0.05$, Kruskal-Wallis); B, hCG, however, decreased the expression of 11βHSD type 2 ($P < 0.05$, Kruskal-Wallis), whereas in the presence of aminoglutethimide inhibition of type 2 was prevented; C, similar to that of 11βHSD type 1, hCG increased GR expression in luteinized granulosa cells ($P < 0.0001$, ANOVA), whereas the addition of aminoglutethimide had no effect; D, hCG stimulated an increase of progesterone ($P < 0.0001$, ANOVA), and this increase was blocked by the addition of aminoglutethimide; E, hCG increases 11-oxoreductase activity, which acts to generate cortisol ($P < 0.05$, *t* test).

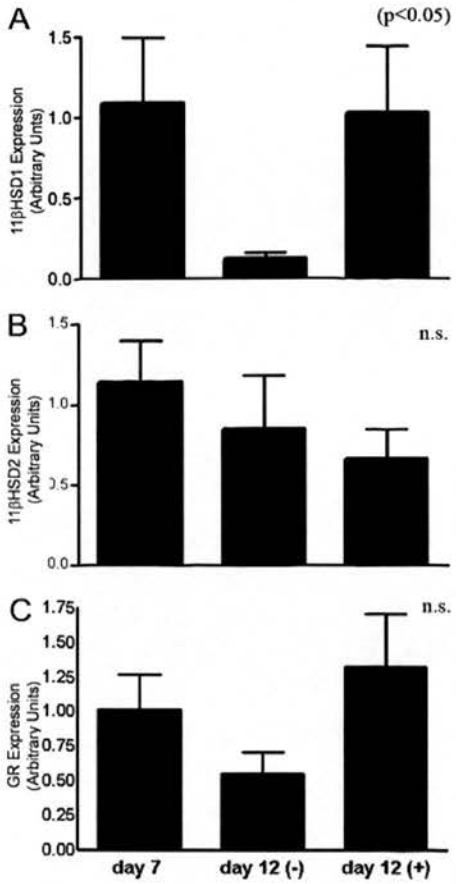

FIG. 3. Chronic manipulation with hCG in cultures of luteinized granulosa cells designed to mimic the luteal phase involved low-dose hCG stimulation until d 7, which was either replaced by maximal-dose hCG on d 12 (+) or removal of hCG on d 12 (−). A, Withdrawal of hCG on d 12 down-regulated the expression of 11βHSD type 1 ($P < 0.05$, Kruskal-Wallis), whereas expression was maintained in the presence of hCG; B, expression levels of 11βHSD type 2 remained unchanged ($P > 0.05$, Kruskal-Wallis) by hCG withdrawal or maintenance; C, the expression of GR demonstrated a tendency to decrease with hCG withdrawal and maintain levels with hCG ($P < 0.05$, ANOVA). n.s., No significant difference.

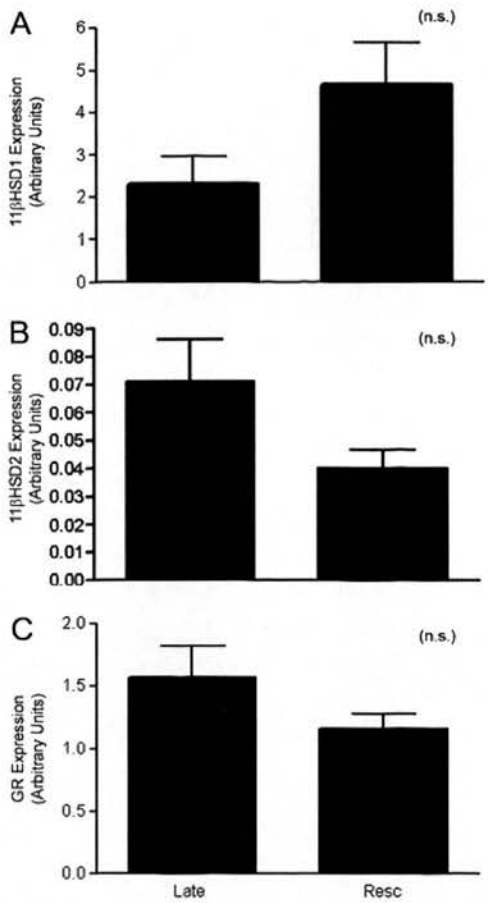

FIG. 4. Modulation of 11βHSDs and GR in human corpora lutea *in vivo* suggests a tendency for cortisol regeneration during hCG rescue. A, The relative expression levels of 11βHSD type 1 tended toward an increase during hCG-induced luteal rescue compared with that of the late luteal phase; B, conversely, the opposite was true for 11βHSD type 2, whereby expression levels tended toward a decline during hCG-induced luteal rescue compared with the late luteal phase; C, expression of GR remained relatively unchanged in the presence or absence of hCG. n.s., No significant difference.

early pregnancy (2). *In vivo*, the effects of hCG did not quite reach significance ($P > 0.05$, *t* test), although there was a clear differential effect on the expression of 11βHSD type 1 and 11βHSD type 2 (Fig. 4). Exogenous hCG tended to up-regulate 11βHSD type 1 (Fig. 4A) but tended to down-regulate 11βHSD type 2 (Fig. 4B). There were no specific effects of hCG on the expression of GR in the corpus luteum of women (Fig. 4C). These data suggest that the differential regulation of cortisol metabolism in luteinized granulosa cells *in vitro* may also occur in the human corpus luteum *in vivo*.

The effect of RU486 on primary cultures of luteinized granulosa cells

Because hCG seems to promote the generation and action of cortisol, we hypothesized that cortisol may function as a local luteotropic factor during luteal rescue and that cortisol withdrawal may have a role during luteolysis. We therefore investigated whether cortisol could function as a survival factor in cultures of luteinized granulosa cells. The cortisol and progesterone antagonist RU486 reduced the number of luteinized granulosa cells in culture ($P < 0.001$, ANOVA) after 7 d treatment (Fig. 5). This effect was not seen when progesterone synthesis was inhibited by aminoglutethimide (Fig. 5). This decrease in cell number in the presence of RU486 could be prevented by the addition of cortisol ($P > 0.05$, ANOVA) so that cell numbers were no different from controls (Fig. 5). Conversely, the addition of saturating concentrations of progesterone or hCG in the presence of RU486 did not increase the cell number beyond that of RU486 alone ($P > 0.05$, ANOVA) (Fig. 5). These data crudely suggest that cortisol may have direct actions on luteinized granulosa cells *in vitro*.

The effect of cortisol on nonsteroidogenic cells

As well as the steroidogenic cells of the corpus luteum, GR can be localized to nonsteroidogenic cells. This suggests that cortisol has the potential to function as a paracrine regulatory

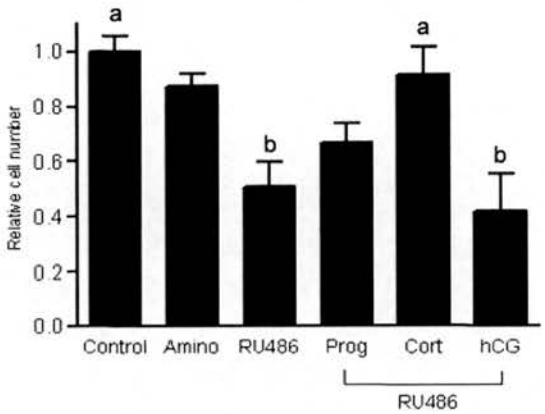

FIG. 5. The effect of RU486 and steroids on luteinized granulosa cell survival after 7 d in culture. Culturing the cells with aminoglutethimide had no effect on the number of cells remaining after 7 d ($P > 0.05$, ANOVA), whereas RU486 reduced cell numbers by 50% ($P < 0.001$, ANOVA). The addition of progesterone to RU486 did not return cell numbers to control levels ($P < 0.05$, ANOVA), whereas the addition of cortisol increased cell numbers ($P < 0.05$, ANOVA) to control levels ($P > 0.05$, ANOVA). The addition of hCG to RU486 had no effect ($P > 0.05$, ANOVA) with numbers less than controls ($P < 0.01$, ANOVA) and RU486 with cortisol ($P < 0.01$, ANOVA). *Different letters* represent significant differences.

factor. We therefore investigated the effect of cortisol on our novel primary cultures of ovarian fibroblast-like cells derived from the luteinizing follicle (20, 23). These cells secrete MMP-2 that is regulated in a paracrine manner by hCG through intermediary molecules (18). *In vivo*, hCG inhibits the expression of luteal fibroblast MMP-2 ($P < 0.05$, *t* test) (Fig. 6A). *In vitro*, our fibroblast-like cells express MMP-2 mRNA and proteolytic activity. The addition of exogenous cortisol inhibited both the expression ($P < 0.001$, *t* test) and activity ($P < 0.0001$, *t* test) of MMP-2 (Fig. 6, B and C). This suggests that cortisol may have the potential to have relevant effects on neighboring nonsteroidogenic cells.

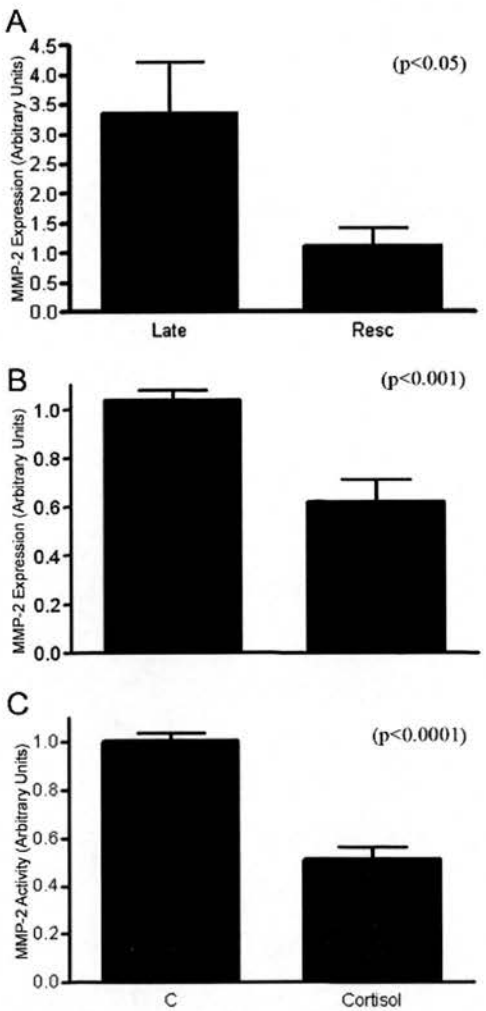

FIG. 6. MMP-2 expression and activity appear to be modulated by glucocorticoids in primary cell cultures of fibroblast-like cells. A, The expression of MMP-2 was down-regulated during hCG-induced luteal rescue in human corpora lutea ($P < 0.05$, *t* test); B, primary cell cultures of fibroblast-like cells treated with cortisol mirror the effect of hCG during luteal rescue because cortisol inhibits MMP-2 expression ($P < 0.001$, *t* test); C, gelatin zymography further confirms that cortisol reduces MMP-2 activity ($P < 0.0001$, *t* test) in these fibroblast-like cells from the luteinizing follicle.

Discussion

We hypothesized that locally produced cortisol may be involved in regulating tissue remodeling during luteolysis. We have shown that the human corpus luteum expresses the enzymes required to increase or decrease the local availability of cortisol and the receptor pathways to respond to glucocorticoids. We have combined studies in human corpora lutea with primary human cell culture models to show that the expression of the 11βHSD enzymes that control the local availability of cortisol is hormonally regulated. Although, as discussed previously, luteinized granulosa cells *in vitro* are not a perfect representation of granulosa-lutein cells *in vivo* (23), we believe that the *in vitro* and *in vivo* paradigms are complementary. Both paradigms suggest that in women, there is a change in 11βHSD isoforms across the luteal phase that is associated with the functional state of the gland. This is complementary to the only other study investigating 11βHSD in the corpus luteum that suggested there may be more 11βHSD type 2 in the regressing rat corpus luteum (17).

It is uncertain what role cortisol has in the human corpus luteum. Roles for active glucocorticoids in the ovary have certainly been proposed and described during folliculogenesis and ovulation (11). Previous experiments in our laboratory demonstrated that hCG has the ability to regulate many different cell types and their molecular functions within the corpus luteum and highlighted the important role for locally produced intermediate regulatory molecules (18, 20, 21, 23). Herein, we propose that glucocorticoids may be regulatory molecules with signaling roles within the corpus luteum. We believe that hCG promotes the generation of active glucocorticoids that have a luteotropic role in the human corpus luteum such that the structural remodeling associated with luteolysis is inhibited and maintenance of early pregnancy is facilitated. Similarly, removal of local cortisol may facilitate luteal regression in the absence of hCG.

The 11βHSDs catalyze the interconversion of active (cortisol) and inactive (cortisone) glucocorticoids by isoforms type 1 and 2, respectively. The temporal and spatial expres-

sions of both isoforms have been documented in the ovary of other species (24–27). It is well established that 11βHSD type 2 is the predominant isoform during the follicular phase of the ovarian cycle, localized to the nonluteinized granulosa cells before the LH surge (26). This is an important concept because too much cortisol during the follicular phase is reported to disrupt FSH-stimulated granulosa cell development/function (and presumably estradiol production) (28), which would consequently inhibit successful folliculogenesis. In addition, during folliculogenesis, the predominant steroid is estradiol, and unlike progesterone (as discussed below), estradiol has a very low affinity for cortisol-binding protein (CBP) (29) and thus will not displace cortisol. Because it has also been reported that estradiol can increase the hepatic production of CBP (30), another mechanism may exist during the follicular phase to lower free cortisol. It seems that in the follicular phase, local cortisol generation and action tends to be inhibited.

This is not the case, however, in the periovulatory period. Once the dominant follicle is exposed to the midcycle preovulatory gonadotropin surge, the predominant isoform switches from type 2 to type 1 11βHSD (26, 27). This phenomenon can also be seen by a rise of free cortisol that is 50 times higher in follicular fluid after the LH surge (10, 31), indicative that expression levels of the 11βHSD enzymes in the ovary are an accurate measure for the direction of glucocorticoid biosynthesis. Indeed, it has been suggested that this process is involved in facilitating fertility. Some studies report that IVF patients with a higher cortisol to cortisone ratio in their follicular fluid have greater pregnancy success rates (32, 33), although others do not agree (31, 34). It is likely, however, that the switch in isoforms around ovulation has effects on the oocyte as well as regulating the inflammatory reaction associated with follicular rupture and its resolution (6). Such findings further support the hypothesis that 11βHSD enzyme activities in the human ovary are developmentally and hormonally regulated (27).

The expression of 11βHSD type 1 in luteinizing granulosa cells is maintained in the granulosa lutein cells of the corpus luteum. We also detected 11βHSD type 2 in the corpus luteum. Type 2 11βHSD mRNA expression and protein has previously been reported to be very low or undetectable in freshly isolated luteinized granulosa cells of both rats and humans (26, 35–37). This suggests that as the corpus luteum is formed there may be a recovery of 11βHSD type 2 expression. Indeed, all our cultures of luteinized granulosa cells expressed 11βHSD type 2, and they had been cultured for at least 7 d before analysis. Although 11βHSD type 1 remains the most abundant isoform in luteinized granulosa cells in culture and in the corpus luteum, both isoforms are expressed. hCG inhibited the expression of 11βHSD type 2 *in vitro* and tended to do the same *in vivo*. In contrast, hCG stimulated 11βHSD type 1 expression acutely and in prolonged cultures and tended to do the same *in vivo*. The regulation of 11βHSD type 1, like in the periovulatory period, seems to a direct effect of hCG signaling. However, as the inhibition of 11βHSD type 2 by hCG was blocked when progesterone synthesis was inhibited, progesterone rather than hCG may be involved in the inhibition of 11βHSD type 2 expression. This finding supports a similar experiment in granulosa cells by Thurston *et al.* (37) and demonstrates the same principles of progesterone actions observed in the kidney and placenta (38–40) whereby progesterone inhibits 11βHSD type 2. Indeed, because progesterone receptor expression is down-regulated as the corpus luteum matures, this may be a mechanism for the reemergence of steroidogenic cell 11βHSD type 2 expression in the corpus luteum (22).

The effect of hCG on 11βHSD type 1 expression was mirrored by 11-oxoreductase activity levels and the generation of cortisol from cortisone. We are unable, however, to assess cortisol metabolism in the late luteal and rescued corpus luteum to confirm this *in vivo*. Although we believe that changes in the expression of different 11βHSD isoforms in tissues informs the direction of cortisol/cortisone metabolism (27), it is clear that 11βHSD type 1 has the potential to act as a bidirectional enzyme (given the appropriate coenzyme environment) (41). However, previous detailed studies on the direction of metabolism in luteinized granulosa cells (27, 37), ovarian surface epithelial cells (8, 9, 42), and other tissues (43–45) as well as the phenotype of 11βHSD knockout mice (46) have suggested that *in vivo* 11βHSD type 1 preferentially generates cortisol. However, as pointed out by Jonas *et al.* (47), recent studies have established that the net direction of 11βHSD type 1 is dependant upon the cell availability of hexose-6-phosphase-dependent NADPH, which may be different in highly steroidogenically active tissues and the direction of cortisol metabolism *in vivo* remains to be studied.

The human corpus luteum has the potential to respond to locally generated cortisol because it expresses nuclear GR. Indeed, GR has been localized previously to many cell compartments in the ovary (36). Although the primary receptor for cortisol is GR, cortisol also has a high affinity for the mineralocorticoid receptor. We did not analyze the expression of mineralocorticoid receptor, but it has been reported to be expressed in the ovary (36). We have localized the expression of GR to the nuclei of steroidogenic cells. These cells of the human corpus luteum are also reported to express other important nuclear steroid receptors such as estrogen and progesterone receptors (48–50). It is unclear whether other steroids can influence glucocorticoid signaling by receptor-dependent mechanisms, but it is likely that cortisol has direct effects on the cells expressing the 11βHSD enzymes involved in its synthesis and metabolism.

It is not clear whether the expression of GR in the corpus luteum is regulated. We have shown that steroidogenic cell progesterone receptors are differentially regulated in the corpus luteum across the luteal phase (22), although their role has not yet been elucidated (51). In contrast, we could see no obvious changes in steroidogenic cell GR immunostaining across the luteal phase. Indeed, in the endometrium, where glandular progesterone receptor expression in the secretory phase shows changes similar to that in luteal steroidogenic cells (22, 52), there was no change in GR expression across the functional menstrual cycle (53). However, *in vitro* hCG tended to up-regulate GR expression similar to its effects on 11βHSD type 1. Whether GR expression in corpora lutea is hormonally regulated but masked by detection methods is

not entirely clear. What is clear is that multiple cell types in each corpus luteum express nuclear GR.

Luteal endothelial cells and macrophages express nuclear GR. Protein colocalization of GR with CD31 demonstrates specific nuclear staining of endothelial cells. The effect of cortisol on luteal endothelial cells is not clear, but glucocorticoids have been shown to suppress angiogenesis (54), and this is most notably due to the suppression of vascular endothelial growth factor action (54, 55). In the human corpus luteum, however, there continues to be some angiogenesis stimulated by hCG during luteal rescue (56) in response to the marked up-regulation of vascular endothelial growth factor after exogenous hCG (57). Immune cells, most notably macrophages (CD68-positive cells), which also express GR, accumulate in the corpus luteum during luteolysis and show a marked reduction in number during hCG-induced luteal rescue (1). The role of cortisol in luteal macrophage accumulation and action is not clear, but because glucocorticoids are known to regulate cytokine signaling and macrophages in both health and disease (58), it may affect the immune cell-mediated processes during luteolysis. Luteal myofibroblasts are known to express macrophage chemoattractant protein-1 (59), and these cells also express nuclear GR.

When considering a role for glucocorticoids in the corpus luteum, it is very important to establish the relationship between cortisol and the marked excess of structurally related progesterone. Cortisol exists in one of two forms, bound and free, that regulate its bioavailability. In most systems, the majority of the steroid is bound to plasma proteins (notably CBP) with only a fraction free and unbound (12). Although CBP has the highest affinity for cortisol of all the binding globulins, other steroids such as progesterone and particularly 17OH-progesterone have high binding affinities to CBP (12, 29). Therefore, very high concentrations of progesterone and progesterone metabolites (such as in the corpus luteum) will displace cortisol from CBP, that acts as a buffer reservoir, resulting in the environment becoming enriched with free cortisol. This scenario is known as the free hormone hypothesis (12, 29) and predicts that the bioactivity of cortisol is proportional to free hormone concentrations and not total concentration, which includes the protein-bound fraction. This is an important paradigm to consider because the concentration of free biologically active cortisol in preovulatory follicular fluid is 10 times greater than that of serum (31). It is likely that, because of high local progesterone concentrations, rather than being mainly bound to CBP in the corpus luteum, locally generated cortisol is more likely to be free and functional at lower concentrations.

It is still not clear what effects cortisol has on luteal cells. We hypothesized that it may affect the survival of luteinized granulosa cells. Our observations and those of others (60) suggested that RU486 reduced survival of these cells in culture. We used simple cell counting to document the effect on RU486 on cell survival. Because cells treated with the progesterone synthesis disruptor aminoglutethimide, which blocks P450 side-chain cleavage, showed no change in their morphology or viability, we tested whether the effect of RU486 could be reversed by hCG or exogenous progesterone. Unlike a previous study using human luteinized granulosa cells, under slightly different conditions (60), we could not fully reverse the RU486 effects using progesterone. However, we were able to do so using cortisol. It is not clear whether this effect is at the level of the receptor because the concentration of cortisol would not be expected to fully displace the RU486. It may be that cortisol affects the cell susceptibility to RU486 in different ways. There may be specific effects of RU486 not mediated by hormone antagonism. Direct actions of RU486 have been reported in ovarian epithelial cancer cells and human endometrium, whereby it down-regulated molecules involved in signal transduction pathways by cytokines, growth factors, and other physiological stimuli that control cell functions (61). Indeed, in cultured luteinized granulosa cells, cortisol and dexamethasone offer protection against serum deprivation and induced apoptosis by bcl-2 and TNF-α (62, 63) through mechanisms that include stabilization of the actin cytoskeleton (64). Whatever the mechanism of action, our results crudely suggest that cortisol may have direct effects on luteal steroidogenic cells. Indeed, if cortisol has any direct genomic effects *in vivo*, they are more likely to promote, rather than inhibit, steroidogenic cell survival and function.

If cortisol does have luteotropic actions, it may also have specific actions on the nonsteroidogenic cells of the corpus luteum that are key regulators of tissue remodeling during luteolysis. Luteal fibroblasts are the main source of MMP-2, a key proteolytic enzyme involved in tissue remodeling associated with luteolysis in women (2) and in many other species (65–67). Both primates (67) and women (2) show maximal MMP-2 expression in the late luteal phase. However, during maternal recognition of pregnancy, MMP-2 production is considerably reduced (2), suggestive that hCG is regulating the enzymatic expression through intermediate molecules (18). Furthermore, it is tempting to speculate that active glucocorticoids may also prevent luteolysis by inhibition of intraluteal prostaglandin synthesis (68). Although this is an attractive suggestion, the actual role of prostaglandins in the human corpus luteum remains elusive.

In the present study, our novel findings suggest that cortisol may be involved in paracrine interactions that control tissue remodeling. Recently, an *in vitro* study from our laboratory modeling the human corpus luteum has shown that activin A is a paracrine factor secreted from luteinized granulosa cells that may up-regulate fibroblast MMP-2 secretion (23) in the absence of hCG. In contrast to activin A (23), cortisol treatment of luteal fibroblast-like cells in culture resulted in a reduction in the production of MMP-2, a pattern reflecting MMP-2 expression in exogenous hCG-rescued luteal tissue (2). Indeed, glucocorticoids decreased MMP-2 activity in rat aortic smooth muscle cells (69) and in a fibrosarcoma cell line (70). It seems that the nonsteroidogenic cells forming the corpus luteum have the ability to directly respond to cortisol. If these effects do occur *in vivo*, it is likely that cortisol tends to inhibit rather than stimulate the remodeling associated with luteolysis and may therefore be considered to be luteotropic in nature.

We hypothesized that during luteolysis, more cortisol is generated in the local environment, consequently preventing aberrant scarring to the tissue. It is clear, however, from the present study that luteolysis is not associated with an in-

crease in cortisol, and the opposite is true. We have shown that hCG tends to generate cortisol by up-regulating 11βHSD type 1 and down-regulating 11βHSD type 2. We have shown that the corpus luteum has the potential to react to this cortisol and that the effect on steroidogenic and neighboring cells tends to be luteotropic rather than luteolytic. In summary, our observational and interventional *in vivo* and *in vitro* models have generated results that suggest that cortisol tends to be withdrawn during luteolysis and maintained during luteal rescue. Glucocorticoids may have a role in the local luteal regulation of maternal recognition of pregnancy in women.

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